

ARNOLD & PORTER

202.942.5000
202.942.5999 Fax

555 Twelfth Street, NW
Washington, DC 20004-1206

RECEIVED
SEP 25 PM 4:26

September 25, 2003

Examiner Fozia Hamud
U.S. Patent & Trademark Office
Crystal Mall One – 7th Floor
1911 South Clark Place
Arlington, VA 22202

via Hand-Delivery

Re: U.S. Patent Application No. 09/202,455
Filed: December 15, 1998
Title: **An OCIF-Binding Molecule (OBM), Nucleic Acid Encoding, and
Process for Producing the Same (As Amended)**
Applicants: Kyoji YAMAGUCHI *et al.*
Atty. Docket No.: 16991.011

Dear Examiner Hamud:

Pursuant to our telephone conversation on Wednesday, September 24, 2003, please find enclosed courtesy copies of the following documents, as previously filed in the U.S. Patent and Trademark Office on August 15, 2003:

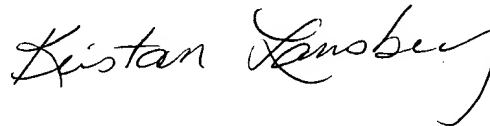
1. a Transmittal letter;
2. a Petition for Extension of Time;
3. a Request for Entry of Substitute Specification;
4. a clean copy of the substitute specification (Tab 3);
5. a computer-generated marked-up copy of the specification (Tab 4);
6. an English translation of PCT/JP98/01278 (Tab 5); and
7. a return postcard.

ARNOLD & PORTER

Examiner Fozia Hamud
Atty. Docket No.: 16991.011
Page 2

If you have any questions or need additional materials, please do not hesitate to contact me directly at (202) 942-5186.

Respectfully submitted,

A handwritten signature in cursive script, reading "Kristan Lansbery". The signature is written in dark ink and is positioned above the printed name.

Kristan L. Lansbery (Reg. Agent No. 53,183)

Enclosures

cc: David R. Marsh (w/o encls.)

COPY

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:

Kyoji YAMAGUCHI *et al.*

Appln. No.: 09/202,455

U.S. Filing Date: December 15, 1998

For: *An OCIF-Binding Molecule (OBM),
Nucleic Acid Encoding, and Process
for Producing the Same (As Amended)*

Art Unit: 1647

Examiner: Fozia M. HAMUD

Atty. Docket: 16991.011

Confirmation No.: 2918

Request for Entry of Substitute Specification

Mail Stop AF

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

Sir:

Submitted herewith is a substitute specification for the above-referenced application, for which entry is respectfully requested.

The attached substitute specification (please see Tab 3) amends the originally filed specification to be based on the attached English Translation of PCT/JP98/01728 (submitted herewith; please see Tab 5) and the Amendment and Response to Restriction Requirement filed on January 3, 2000 and August 10, 2000. Support for the foregoing substitute specification may be found throughout the specification as filed, in the original claims, and in the attached English Translation of PCT/JP98/01728. No new matter enters by way of this submission. Pursuant to 37 C.F.R. § 1.125, a computer-generated marked-up version of the substitute specification is also submitted herewith (please see Tab 4). Applicants respectfully request entry of the substitute specification (Tab 3). *See* MPEP § 608.

If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application or if the Examiner has any questions regarding this application, the Examiner is invited to contact Applicants' undersigned representative at (202) 942-5186.

In the event that extensions of time beyond those petitioned for herewith are necessary to prevent abandonment of this patent application, then such extensions of time are hereby petitioned. Applicants do not believe that any additional fees are due in conjunction with this filing. However, if any fees under 37 C.F.R. §§ 1.16 or 1.17 are required in the present application, including any fees for extensions of time, authorization to charge such fees is given in the accompanying transmittal letter.

Respectfully submitted,



David R. Marsh (Reg. Attorney No. 41,408)
Dawn G. Krosnick (Reg. Attorney No. 44,118)
Kristan L. Lansbery (Reg. Agent No. 53,183)

DATE: August 15, 2003

ARNOLD & PORTER
555 Twelfth Street, N.W.
Washington, D.C. 20004-1206
(202) 942-5000 telephone
(202) 942-5999 facsimile

COPY

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

03 SEP 23 PM 4:25

In re application of:

Kyoji YAMAGUCHI *et al.*

Group Art Unit: 1647

Appln. No.: 09/202,455

Examiner: F. Hamud

Filing Date: December 15, 1998

Atty. Docket No.: 16991.011

For: *An OCIF-Binding Molecule (OBM),
Nucleic Acid Encoding, and Process
for Producing the Same (as
amended)*

Confirmation No.: 2918

Petition For Extension of Time Under 37 C.F.R. § 1.136

Mail Stop AF

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

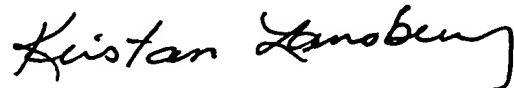
Sir:

It is hereby requested that the period for submitting an Appeal Brief be extended an additional two months from June 15, 2003 to August 15, 2003 by the filing of this Petition and fee payment.

The appropriate fee (37 C.F.R. §1.17(a)) is believed to be \$1,040.00 for a fourth and fifth month extension of time for a large entity. Applicants request that this fee be charged to Deposit Account No. 50-2387. **Attention is directed to the fact that the fee of \$930.00 for a three month extension of time was previously paid on June 13, 2003.** Further, if additional extensions of time under 37 C.F.R. § 1.136 other than those provided for herewith are required to prevent abandonment of the present patent application, then such extensions of time are hereby petitioned.

The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 50-2387 referencing docket number 16991.011.

Respectfully submitted,



David R. Marsh (Attorney Reg. No. 41,408)
Dawn G. Krosnick (Attorney Reg. No. 44,118)
Kristan L. Lansbery (Agent Reg. No. 53,183)

Date: August 15, 2003

ARNOLD & PORTER
555 12th Street, N.W.
Washington, D.C. 20004
202.942.5000 telephone
202.942.5999 facsimile

3

AN OCIF-BINDING MOLECULE (OBM), NUCLEIC ACID ENCODING, AND
PROCESS FOR PRODUCING THE SAME

5

TECHNICAL FIELD

The present invention relates to a novel protein (OCIF binding molecule; hereinafter it may be referred to as "OBM"), which binds osteoclastogenesis inhibitory factor, and a production method thereof. In addition, the present invention also relates to
10 DNA which encodes the protein, a protein having an amino acid sequence encoded by the DNA, a method for genetically producing the protein, and a pharmaceutical composition comprising the protein.

The present invention also relates to a methods of screening for: a substance which controls expression of the protein, a substance which inhibits or modifies the
15 biological activity of the protein, or a receptor which binds the protein and transmits the activity thereof, methods of using the protein or the DNA; the substances obtained by these methods; and pharmaceutical compositions comprising the obtained substances. In addition, the present invention also relates to an antibody to the protein, a method for the production thereof, a method for measuring the protein with the antibody, and an agent comprising the
20 antibody.

BACKGROUND ART

Bone metabolism depends on the overall activity of osteoblasts responsible for bone formation, and osteoclasts, responsible for bone resorption. It is assumed that bone
25 metabolism abnormality is caused due to loss of balance between bone formation and bone resorption. As diseases involving bone metabolism abnormality, osteoporosis, hypercalcemia, bone Paget's disease, renal osteodystrophy, rheumatoid arthritis and osteoarthritis are known. A representative of these bone metabolism abnormality diseases is osteoporosis. This disease occurs when bone resorption by osteoclasts exceeds bone
30 formation by osteoblasts and is characterized by equal decrease in bone calcareous substances and bone matrix. The mechanism for crisis of this disease is not yet fully clarified, while it is a disease with pain in bone and bone fracture due to the increased fragility of bone. Along with an increase in the population of aged people, this disease causes aged people to fracture bone, resulting in confinement in bed. This disease is already
35 a social problem, so that medicaments for treating the disease are urgently needed to be developed. It is expected that osteopenia due to bone metabolism abnormality can be treated

by stimulating bone formation, inhibiting bone resorption, or improving the balance between them. That is, bone formation is expected to be stimulated by promoting the growth, differentiation and functions of osteoblasts, which are responsible for bone formation, suppressing the differentiation of osteoclast precursor cells to osteoclasts and maturation thereof, or suppressing osteoclast function such as bone-resorbing activity. At present, hormones, substances of low molecular weight or physiologically active proteins having such activity are being studied and developed.

As agents for treating bone-related diseases and shortening treatment periods thereof, a calcitonin-containing formulation, the active-form of vitamin D₃-containing formulation, hormone (estradiol, ipriflavone, vitamin K₂) -containing formulation and bisphosphonate-based compound are already clinically available. Furthermore, to develop medicaments with less side effects and excellent effectiveness, clinical trials of the active-form of vitamin D₃ derivatives, estradiol derivatives, and bisphosphonate-based compounds of the second or third generation have been held.

However, since such methods for treatment using these drugs are not necessarily sufficient in effectiveness and results of treatment, novel medicaments that are safer and have higher effectiveness have been expected to be developed. Moreover, among medicaments used in treatment of bone metabolism diseases, there are those which can be used only for treating a restricted kind of disease due to the side effects thereof. In addition, at present, to treat bone metabolism diseases such as osteoporosis, treatment with combined use of more than one medicament is currently usual. From such a point of view, a medicament having different action mechanisms from those of the conventional ones with higher effectiveness and less side effects have been expected to be developed.

As described above, cells responsible for bone metabolism are osteoblasts and osteoclasts. It is known that these cells closely interact with each other, and this phenomenon is regarded as coupling. That is, it has been reported that the differentiation and maturation of osteoclasts are stimulated or suppressed by cytokines, interleukins 1 (IL-1), 3 (IL-3), 6 (IL-6) and 11 (IL-11), granulocyte-macrophage colony-stimulating factors (GM-CSF), macrophage colony-stimulating factors (GM-CSF), interferon gammas (IFN- γ), tumor necrosis factors α (TNF- α), transforming growth factors β (TGF- β) and the like, which are secreted from osteoblast-like stroma cells (Raisz: Disorders of Bone and Mineral Metabolism, 287 to 311, 1992; Suda *et al.*: Principles of Bone Biology, 87 to 102, 1996; Suda *et al.*: Endocrine Reviews, 4, 266 to 270, 1983, Lacey *et al.*: Endocrinology, 136, 2369 to 2376, 1995). It is known that osteoblast-like stromal cells play an important role in differentiation and maturation of osteoclasts and expression of mature osteoclast function, such as bone resorption, through intercellular binding to immature precursor cells of

osteoclasts or (mature) osteoclasts. As a factor involved in osteoclastogenesis by the intercellular binding, a molecule known as osteoclast differentiation factor (ODF) (Suda *et al.*: Endocrine Rev. 13, 66 to 80, 1992; Suda *et al.*: Bone 17, 87S to 91S, 1995) which is expressed on the membrane of the osteoblast-like stromal cell is predicted. According to this assumption, a receptor for ODF exists in the osteoclast precursor cell. However, ODF and this receptor are not yet either purified or identified, and there are no reports on their characteristics, action mechanisms or structures. As just described, the mechanism for differentiation and maturation of osteoclasts has not been fully understood yet, and it is expected that full understanding of that mechanism will significantly contribute not only to the field of experimental medicines but also to developments of novel agents for treating bone metabolism abnormality, based on the novel action mechanism.

Under the circumstances, the present inventors have made intensive studies and found osteoclastogenesis inhibitory factors (OCIF) in the culture solution of human fetal lung fibroblasts IMR-90 (ATCC CCL186) (WO 96/26217).

Then, the present inventors succeeded in DNA cloning of OCIF, production of a recombinant OCIF using an animal cell, and confirmation of *in vivo* medicinal virtues (bone metabolism improving effect) of the recombinant OCIF. OCIF is expected as a medicament that has higher effectiveness and causes less side effects than the conventional one and can prevent and treat diseases associated with bone metabolism abnormality.

DISCLOSURE OF THE INVENTION

The present inventors have intensively searched for the existence of a protein that binds to osteoclastogenesis inhibitory factor OCIF by using OCIF. As a result, the inventors have found that OCIF binding protein is specifically expressed on an osteoblast-like stromal cell cultured in the presence of bone resorption factors such as the active-form of vitamin D₃ and parathyroid hormone (PTH). Furthermore, as a result of studying the characteristics and physiological functions of OCIF binding protein, the protein was found to have biological activity as a so-called osteoclast differentiation and maturation factor, associated with differentiation of immature osteoclast precursor cells to osteoclasts and maturation thereof. The present invention has been completed based on this finding. Moreover, as a result of further studying the protein of the present invention, the present inventors have found that the novel membrane protein is an important protein which leads the differentiation and maturation of immature osteoclast precursor cells to osteoclasts by osteoblast-like stromal cells in a co-culture system of the osteoblast-like stromal cells and spleen cells. The successful identification, isolation and purification of the protein as a factor which supports and promotes the differentiation and maturation of osteoclasts in the present

invention enables a screening of a novel agent for treating bone metabolism abnormality, based on a mechanism for bone metabolism in a living subject, using the protein of the present invention.

Therefore, an object of the present invention is to provide a novel protein (OCIF binding molecule; OBM), which binds osteoclastogenesis inhibitory factor (OCIF), and a method for the production thereof. Another object of the present invention is to provide DNA which encodes the protein, a protein having an amino acid sequence encoded by the DNA, a method for genetically producing the protein, and a pharmaceutical composition comprising the protein. Furthermore, another object of the present invention is to provide an agent for preventing and/or treating bone metabolism abnormality comprising the protein. Moreover, another object of the present invention is to provide: a method of screening for: a substance which controls expression of the protein, a substance which inhibits or modifies the biological activity of the protein, or a receptor which binds the protein and transmits the activity of the protein; a method of using the protein and DNA thereof; a substance obtained by that method; and pharmaceutical compositions comprising the obtained substance. Furthermore, another object of the present invention is to provide an antibody to the protein, a method for production thereof, a method for measuring the protein using the antibody, and a medicament (agent; pharmaceutical composition) comprising the antibody.

The protein of the present invention shows the following physicochemical properties and biological activity. That is, (a) the protein specifically binds osteoclastogenesis inhibitory factor (OCIF) and has high affinity (a dissociation constant, a Kd value, on a cell surface, is not larger than 10^{-9} M); (b) the protein shows a molecular weight of about 30,000 to 40,000 as measured by SDS-polyacrylamide electrophoresis under non-reducing conditions, and shows an apparent molecular weight of about 90,000 to 110,000 when crosslinked with a monomer-type OCIF; and (c) the protein has an activity to support and promote the differentiation and maturation of osteoclasts in a co-culture of mouse osteoblast-like stromal cells and mouse spleen cells in the presence of bone resorption factors such as the active-form of vitamin D₃ and parathyroid hormones (PTH).

As a representative *in vitro* culture system for osteoclastogenesis, a co-culture system of mouse-derived osteoblast-like stromal cell line, ST2, and mouse spleen cells in the presence of the active-form of vitamin D₃ or PTH is well known. The cells that express the protein of the present invention can be obtained by examining the binding ability of a mouse osteoblast-like stromal cell or mouse spleen cell cultured in the presence or absence of the active-form of vitamin D₃ to OCIF. The protein of the present invention is identified as a protein which is specifically induced on an osteoblast-like stromal cell cultured in the presence of bone resorption factors such as the active-form of vitamin D₃ or PTH. Further, in

consideration of the following facts that osteoclast formation is inhibited by addition of OCIF to the above co-culture system in the presence of the active-form of vitamin D₃, in a dose-dependent manner within a range of 1 to 40 ng/ml of OCIF; that there is an intimate correlation between change in expression of the present protein induced on the ST2 cells in the presence of the active-form of vitamin D₃ and the change in osteoclast formation with the passage of time; that the amount of the present protein expressed on a ST2 cell corresponds to the intensity of an ability to support osteoclast formation; and that osteoclast formation is completely inhibited by binding of OCIF(s) to the present protein on the ST2 cells, the protein of the present invention is identified as a protein having biological activity (effect) to support and promote the differentiation and maturation of osteoclasts.

The affinity of the protein of the present invention for OCIF can be assessed by labeling OCIF and testing the binding activity of the labeled OCIF to the surface of an animal cell membrane. OCIF can be labeled by a commonly used protein-labeling method such as labeling with a radioisotope or fluorescence labeling. For instance, an example of labeling OCIF with a radioisotope is ¹²⁵I labeling at a tyrosine residue, and labeling methods such as Iodogen method, chloramine T method and enzyme method can be employed thereto. The binding activity of the thus labeled OCIF to the surface of an animal cell membrane can be examined in accordance with a commonly used method, and the amount of nonspecific binding can be measured by adding 100 to 400 times excess amounts of unlabeled OCIF to the medium for the binding experiment. The amount of specific binding of OCIF is calculated by subtracting that of the nonspecific binding from that of total binding. The affinity (for OCIF) of the present protein expressed on a cell membrane is assessed by conducting the test with various amounts of the labeled OCIF and analyzing the amount of the specific binding by Scatchard plot. The determined affinity of the protein of the present invention for OCIF is about 100 to 500 pM. Thus, the protein of the present invention is identified as a protein having such high affinity (the dissociation constant, the K_d value, on a cell membrane is not larger than 10⁻⁹ M) for OCIF. The molecular weight of OBM is measured by use of gel filtration chromatography, SDS-PAGE or the like. To measure the molecular weight more accurately, SDS-PAGE is preferably used, and OBM is identified as a protein having a molecular weight of about 40,000 (40,000 ± 4,000) under reducing conditions.

The protein of the present invention can be obtained from a mouse osteoblast-like stromal cell line, ST2, a mouse fat cell strain PA6, or human osteoblast-like cell lines, or concentrated osteoblast-like cells obtained from mammals such as human, mouse and rat. And, substances that are required to express the protein of the present invention on these cells may be bone resorption factors such as the active-form of vitamin D₃ (calcitriol), parathyroid

hormone (PTH), interleukins (IL)-1, IL-6, IL-11, oncostatin M, and leukemia cell growth inhibiting factor (LIF). As for the amounts of these substances, it is desirable to use the active-form of vitamin D₃ or PTH in an amount of 10⁻⁸ M; the IL-11 and the oncostatin M in amounts of 10 ng/ml and 1 ng/ml, respectively; and the IL-6 in an amount of 20 ng/ml with 500 ng/ml of IL-6 soluble receptor. It is preferable to use cells obtained by culturing mouse osteoblast-like stromal cell line, ST2, in α -MEM containing 10⁻⁸ M of the active-form of vitamin D₃, 10⁻⁷ M dexamethasone and 10% bovine fetal serum for at least one week until the cells become confluent. Thus cultured cells can be removed and collected by using a cell scraper or the like. Moreover, the collected cells can be stored at -80°C until use.

The protein of the present invention can be purified efficiently from a membrane fraction of the thus collected cells. The membrane fraction can be prepared in accordance with a common method used for fractionation of organelles. As a buffer used in preparation of the membrane fraction, various protease inhibitors may be preferably added. Illustrative examples of protease inhibitors to be added include serine protease inhibitors, thiol protease inhibitors, and metalloprotease inhibitors, such as PMSF, APMSF, EDTA, O-phenanthroline, leupeptin, pepstatin A, aprotinin and a soybean trypsin inhibitor. To crush the cells, a Dounce homogenizer, a polythoron homogenizer, an ultrasonicator or the like can be used. The crushed cells can be suspended in a buffer containing 0.5 M sucrose and centrifuged at 600 X g for 10 minutes so as to separate cell nuclei and uncrushed cells as a precipitated fraction. After further centrifugation at 150,000 X g for 90 minutes, a membrane fraction can be obtained as a precipitated fraction. By treating the thus obtained membrane fraction with various surfactants, the protein of the present invention existing on the cell membrane can be solubilized and extracted, efficiently. For solubilization, various surfactants which are conventionally used in solubilization of cell membrane proteins, such as CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), Triton X-100, Nikkol and n-octylglycoside, can be used. The protein of the present invention is preferably solubilized by adding 0.5% CHAPS to the protein and agitating the mixture at 4°C for 2 hours. By centrifuging the thus prepared sample at 150,000 X g for 60 minutes, the solubilized membrane fraction can be obtained as a supernatant.

The protein of the present invention can be purified efficiently from the thus obtained solubilized membrane fraction, using an OCIF-immobilized column, gel or resin. As the OCIF to be used in the immobilization, that isolated from the culture solution of human fetal lung fibroblasts, IMR-90, in accordance with a method described in WO 96/26217 or that obtained by genetic engineering (rOCIF) can be used. This rOCIF can be obtained by incorporating the corresponding human, rat or mouse cDNA into an expression vector in accordance with a common method, expressing the rOCIF with animal

or insect cells such as CHO cells, BHK cells and Namalwa cells, and then purifying it. The thus obtained OCIF shows a molecular weight of about 60 kDa (monomer type) and a molecular weight of about 120 kDa (dimer type). A dimer type OCIF is preferably used in the immobilization. As a gel or a resin for immobilizing OCIF, ECH SEPHAROSE® 4B, EAH SEPHAROSE® 4B, thiopropyl SEPHAROSE® 6B, CNBr-activated SEPHAROSE® 4B, activated CH SEPHAROSE® 4B, epoxy activated SEPHAROSE® 6B, activated thiol SEPHAROSE® 4B (products of Pharmacia Co., Ltd.), TSkgel AF-epoxy TOYOPAL 650, TSkgel AF-amino TOYOPAL 650, TSkgel AF-formyl TOYOPAL 650, TSkgel AF-carboxy TOYOPAL 650, TSkgel AF-Tresyl TOYOPAL 650 (products of Toso Co., Ltd.), amino-CELLULOFINE™ carboxy-CELLULOFINE™, FMP activated CELLULOFINE™, formyl-CELLULOFINE™ (products of Sei Kagaku Kougyo Co., Ltd.), AFFIGEL 10, AFFIGEL 15 and AFFIPREP 10 (products of Bio-Rad Co., Ltd.) are available. Furthermore, as a column for immobilizing OCIF, a HITRAP® NHS-activated column (Pharmacia Co., Ltd.), TSKgel Tresyl-5PW (Toso Co., Ltd.) or the like can be used. As a specific example of a method for immobilizing OCIF with the HITRAP® NHS-activated column (1 ml, Pharmacia Co., Ltd.), the following method is presented. That is, 1 ml of 0.2 M NaHCO₃/0.5 M NaCl (pH 8.3) solution containing 13.0 mg of OCIF is applied to the column and allowed to undergo a coupling reaction at room temperature for 30 minutes. Then, after 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH 4.0) are applied, respectively, 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) is applied again, and then the column is left to stand at room temperature for 1 hour so as to inactivate excess active groups. Thereafter, the column is washed twice with 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH 4.0), and then replaced with 50 mM Tris/1 M NaCl/0.1% CHAPS buffer (pH 7.5). Finally, an OCIF-immobilized column can be prepared. Using the prepared OCIF-immobilized column, gel or resin, the protein of the present invention can be purified efficiently. To prevent the proteolysis of the protein of the present invention, the above various protease inhibitors may also be added to the buffer solution used in purification. After applying the above solubilized membrane fraction to an OCIF-immobilized column or mixing the solubilized membrane fraction with an OCIF-immobilized gel or resin and subsequently stirring the mixture so as to cause the fraction to be adsorbed, the protein of the present invention can be eluted from the OCIF-immobilized column, gel or resin using an acid, various protein-denaturing agents, a cacodylate buffer or the like. To minimize denaturation of the protein of the present invention, it is preferable to neutralize the eluate immediately using a base. As an acid buffer solution used for elution, 0.1 M glycine-hydrochloric acid buffer solution (pH 3.0), 0.1 M glycine-hydrochloric acid buffer solution (pH 2.0) and 0.1 M sodium citrate buffer solution (pH 2.0) can be used, for example.

The purified protein of the present invention can be further purified by use of a method which is conventionally employed in purification of proteins from biological samples, through various purification operations taking advantages of the physicochemical properties of the protein of the present invention. To concentrate a solution of the protein of the present invention, a method which is conventionally used in protein purification process, e.g., ultrafiltration, freeze-drying and salting-out, can be used. Preferably, ultrafiltration based on centrifugation with CENTRICON®-10 (Amicon Co.) and the like is used. Furthermore, as a means of purification, various methods conventionally used in protein purification using ion exchange chromatography, gel filtration chromatography, hydrophobic chromatography, reversed phase chromatography, preparative electrophoresis and the like can be used in combination. More specifically, the protein of the present invention can be purified by concombined use of gel filtration chromatography with SUPEROSE®-12 column (Pharmacia Co., Ltd.) and the like and reverse phase chromatography. Moreover, the protein of the present invention during the purification process can be detected by analyzing activity to bind the immobilized OCIF or by immuno precipitation of OCIF-binding substances with an anti-OCIF antibody followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

The thus obtained protein of the present invention is useful, due to its activity, as medicaments, e.g., as agents for treating bone metabolism abnormality such as osteopetrosis, or experimental and diagnostic reagents.

Furthermore, the present invention relates to DNA which encodes a novel protein (OCIF binding molecule; OBM) which binds osteoclastogenesis inhibitory factor (OCIF), a protein having an amino acid sequence encoded by the DNA, a method for genetically producing a protein which specifically binds OCIF by use of the protein, and an agent for treating bone metabolism abnormality comprising the protein. In addition, the present invention relates to a method for screening a substance which controls expression of OBM, a method for screening a substance which binds OBM and inhibits or modifies an effect thereof, a method for screening a receptor which binds OBM and transmits an effect thereof, and pharmaceutical compositions comprising a substance obtained as a result of these methods for screening.

The novel protein OBM encoded by the DNA of the present invention shows the following physicochemical properties and biological activity. That is, (a) the protein specifically binds osteoclastogenesis inhibitory factor (OCIF), (b) the protein shows a molecular weight of about 40,000 ($\pm 4,000$) as measured by SDS-PAGE under reducing conditions, and shows an apparent molecular weight of about 90,000 to 110,000 when crosslinked with a monomer-type OCIF; and (c) the protein has an activity to support and

promote differentiation and maturation of osteoclasts.

Human osteoclastogenesis inhibitory factor (OCIF) is used as a probe for assessing the properties of OBM in identification of the DNA encoding OCIF binding molecule OBM of the present invention, and can be isolated from the culture solution of human fetal lung fibroblasts, IMR-90, in accordance with WO 96/26217. For isolation and identification of the DNA encoding OBM, recombinant human OCIF, recombinant mouse OCIF, recombinant rat OCIF and the like can also be used. This recombinant OCIF can be obtained by incorporating the corresponding DNA into an expression vector in accordance with a commonly used method, subsequently expressing OCIF with animal or insect cells such as CHO cells, BHK cells and Namalwa cells, and then purifying it.

Methods for cloning a cDNA which encodes the target protein (cDNA cloning) include, a method comprising the steps of determining a partial amino acid sequence of the protein and isolating the target cDNA by hybridization based on a nucleotide sequence corresponding to the amino acid sequence. Another method comprises the steps of constructing a cDNA library with an expression vector, regardless of whether or not the amino acid sequence of the protein is known, subsequently introducing it into cells, and then screening for the presence and absence of expression of the target protein and isolating the desired cDNA (D' Andrea *et al.*: Cell 57, 277 to 285, 1989; Fukunaga *et al.*: Cell 61, 341 to 350, 1990) (expression cloning method). In the expression cloning method, bacterial, yeast, animal cells and the like are selected and used as host cells according to the purpose. For cloning a cDNA which encodes a protein considered to present on the surface of animal cell membrane as in the present invention, animal cells are often used as hosts. Furthermore, hosts with high efficiency for introducing DNA and expressing the introduced DNA are conventionally used. One of the cells having such characteristics is a monkey kidney cell line, COS-7, used in the present invention. Since SV40 large T antigen is expressed in COS-7 cells, plasmids having a SV40 origin of replication are present in the cell as multicopy episome, whereby higher expression than usual can be expected. Moreover, since the maximum expression level is reached within a few days after the introduction of DNA, COS-7 cells are suitable for quick screening. In combination with a plasmid suitable for high expression, this host cell enables an extremely high level of gene expression. The promoter is a factor of a plasmid which has the most significant effects on the amount of gene expression. As a promoter suitable for high level of expression, SR α promoter and cytomegalovirus-derived promoter are often used. Screening methods for cloning the cDNA of the membrane protein include expression cloning, binding method, panning method and film emulsion method.

The present invention relates to DNA, which encodes the protein which

specifically binds OCIF (OBM), obtained by a combination of the expression cloning method and the binding method, the protein expressed therewith, and a screening of a biologically active substance with the DNA or the protein. OBM encoded by the DNA of the present invention can be detected by labeling OCIF and subsequently examining the binding activity of the labeled OCIF to the surface of an animal cell membrane. OCIF can be labeled by a conventional method for labeling protein such as labeling with a radioisotope or fluorescence labeling. An example of labeling OCIF with radioisotope is ^{125}I labeling at tyrosine residues, and specific labeling methods include Iodogen method, chloramine T method and enzyme method. The binding activity of thus labeled OCIF to the surface of an animal cell membrane can be assessed in accordance with a commonly used method. Furthermore, an amount of nonspecific binding can be measured by adding 100 to 400 times excess amount of unlabeled OCIF to the medium for a binding experiment. The amount of specific binding of OCIF is calculated by subtracting that of the nonspecific binding from that of the total binding.

Based on an assumption that a factor, which is involved in differentiation of osteoclasts, interacts with OCIF, the inventors have screened an expression library prepared from the mRNA of a mouse osteoblast-like stromal cell line, ST2, with recombinant OCIF in accordance with the following method in order to separate the protein which binds OCIF. DNA synthesized from the mRNA of ST2 cells was inserted into an expression vector for an animal cell, and they were transduced (transfected) into COS-7 monkey kidney cells. Using ^{125}I -labeled OCIF as a probe, the target protein expressed on the COS-7 cell was screened. As a result, DNA which encodes the protein that specifically binds OCIF could be separated, and then the nucleotide sequence of the DNA which encodes this OCIF binding molecule (OCIF binding molecule; OBM) was determined. Furthermore, it has been found that OBM encoded by the DNA strongly and specifically binds OCIF on the cell membrane.

An example of DNA hybridization under relatively mild conditions in the present invention is that after DNA is transferred to a nylon membrane and fixed in accordance with a common method, it is hybridized with a radio-labeled DNA as a probe in a hybridization buffer at 40 to 70°C for about 2 hours to overnight, and then washed with 0.5 X SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 45°C for 10 minutes. More specifically, after DNA is transferred and fixed to a nylon membrane, HYBOND® N (Amersham Co., Ltd.), in accordance with a conventional method, it is hybridized with a ^{32}P -labeled DNA as a probe in Rapid Hybridization Buffer (Amersham Co., Ltd.) at 65°C for 2 hours, and then washed with 0.5 X SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 45°C for 10 minutes.

As a representative *in vitro* culture system for osteoclastogenesis, a co-culture system of mouse-derived osteoblast-like stromal cell line, ST2, and mouse spleen cells in the

presence of the active-form of vitamin D₃ or PTH is well known. OBM of the present invention is identified as a protein which is specifically induced on an osteoblast-like stroma cell cultured in the presence of bone resorption factors such as an active-form of vitamin D₃ and PTH. Furthermore, since osteoclasts formation is stimulated by adding the protein encoded by the DNA of the present invention to a culture system of mouse spleen cells even in the absence of the active-form of vitamin D₃ or PTH, OBM encoded by the DNA of the present invention is considered to be involved in differentiation and maturation of osteoclasts.

A recombinant OBM can be produced by inserting the DNA of the present invention into an expression vector so as to prepare a plasmid for expressing OBM, and then introducing and expressing the plasmid in various cells and microbial strains. COS-7, CHO, Namalwa and the like can be used as mammalian hosts cells for expression, and *Escherichia coli* (*E. coli*) and the like can be used as bacterial host cells for expression. In such a case, the recombinant OBM can be expressed as a membrane-bound protein using full length of DNA or as a secretory-type or solubilized-type (soluble-type) protein by removing a part of the DNA encoding a membrane-binding domain from the full length. Thus produced recombinant OBM can be purified efficiently in combination with conventional methods used in protein purification such as affinity chromatography using an OCIF-immobilized column, ion exchange chromatography, gel filtration chromatography and the like. The thus obtained protein of the present invention is useful, due to its activity, as medicaments, e.g., as agents for treating bone metabolism abnormality such as osteopetrosis and as experimental or diagnostic reagents.

The protein OBM encoded by the DNA of the present invention enables:

(1) screening of a substance which controls expression of OBM; (2) screening of a substance which specifically binds OBM and inhibits or modifies the biological activity of OBM; and (3) screening of a protein (OBM receptor) which exists on a precursor cell of osteoclasts and transmits the biological activity of OBM; and (4) as well as developments of antagonists and agonists using this OBM receptor. In combinatorial chemistry using the above OBM or

OBM receptor, a peptide library required to identify an antagonist or agonist can be prepared

in accordance with the following specific methods. One of them is a split method (Lam *et al.*; Nature 354, 82 to 84, 1991). In this method, synthetic carriers (beads) are bound to amino acids (units), separately. Then, these synthetic beads are mixed together and divided into an equal number of units, and then bound to the subsequent units. By repeating this operation n times, a library in which n units are bound to the carriers is prepared. Such an

operation allows the synthesis of only one sequence per one group of the carriers. Hence, when a positive carrier group is selected in said method for screening by use of the protein of

the present invention and then the amino acid sequence thereof is determined, a specifically binding peptide can be identified. As another method, a phage display method can be used. In this method, synthetic genes encoding random peptides are expressed using phage. While this method has an advantage that it can archive a larger number of molecules in a library than the above synthetic library, it also has a disadvantage that the kind of peptides per molecules is not as varied because peptides having sequences that phages don't prefer do not exist in the library. In the phage display method, as in the case of the split method, using a screening system with the protein of the present invention, phage specifically binding thereto are concentrated by panning. Thus obtained phage are amplified in *E. coli*, and further, the nucleotide sequence encoding the peptide is determined. Furthermore, when it is desired that a specific peptide having high affinity for OBM or OBM receptor is screened from a peptide library using the screening system of the above (2) or (3), a specific peptide having a very high affinity can be obtained by screening a positive carrier or phage in the co-presence of OCIF or OBM with a change of concentration. For example, screening of a peptide agonist of low molecular weight having an EPO-like activity from a varied peptide library with an erythropoietin (EPO which is a hematopoietic hormone) receptor, analysis of a three-dimensional structure thereof, and the production of a substance (agonist) of low-molecular-weight having an EPO activity through synthesis of organic chemical compounds based on the three-dimensional structure has already been successful (Nicholas *et al.*: Science, 273, 458 to 463, 1996).

Furthermore, the inventors have found that a protein binding OCIF is specifically expressed on an osteoblast-like stromal cell line, ST2, which was cultured in the presence of bone resorption factors such as the active-form of vitamin D₃ and parathyroid hormone (PTH), using osteoclastogenesis inhibitory factor (OCIF). Moreover, the inventors have found that the protein, which is associated with differentiation of immature osteoclast precursor cells to osteoclasts and maturation thereof, has a biological activity as a factor which supports and promotes so-called differentiation and maturation of osteoclasts. After purification of the protein, the physicochemical properties and biological activity of the protein were examined. The inventors have compared the physicochemical properties and biological activity of the recombinant protein OBM by expressing the DNA of the present invention with those of a purified natural-type protein which specifically binds OCIF in order to clarify differences between them. As a result, they have found that (1) each of both proteins is a membrane-bound protein and specifically binds OCIF; (2) they shared a molecular weight of about 40,000 as measured by SDS-PAGE; and (3) they have an apparent molecular weight of about 90,000 to 110,000 when crosslinked with a monomer-type OCIF, which indicates that they have very similar physicochemical properties. An activity to

support and promote differentiation and maturation of osteoclasts was also shared by them as well. Therefore, the possibility that both proteins are identical was suggested. Furthermore, an anti-OBM rabbit polyclonal antibody prepared with the protein (recombinant OBM), which was genetically expressed with the DNA of the present invention and then purified, has cross-reactivity to the purified natural-type protein obtained by the above method and specifically inhibited the binding between said natural-type protein and OCIF, just as it inhibits specific binding between OBM and OCIF. From these results, it is obvious that the recombinant protein OBM expressed with the DNA of the present is identical to the natural-type protein which specifically binds OCIF.

Furthermore, for isolating a gene (cDNA) that encodes a human-derived OCIF binding protein molecule (hereinafter referred to as "human OBM") which specifically binds OCIF and has an activity to support and promote differentiation of mouse spleen cells to osteoclasts and maturation, just as the natural-type or the recombinant mouse OBM does, the inventors have carried out a polymerase chain reaction (PCR) using primers prepared based on the above mouse OBM cDNA and human lymph node-derived cDNA as a template. Thus, the inventors have screened said cDNA library with the obtained human OBM cDNA fragment. As a result, they have succeeded in isolation of the cDNA which encodes the human-derived protein which specifically binds OCIF (human OBM) and determination of the nucleotide sequence of said cDNA. They have found that human OBM encoded by the cDNA strongly and specifically binds OCIF on a cell membrane and has a biological activity to support and promote differentiation of mouse spleen cells to osteoclasts and maturation thereof, just as mouse OBM does. That is, other objects of the present invention are to provide: (1) DNA which encodes human OBM which is a novel human-derived protein which binds osteoclastogenesis inhibitory factor (OCIF; a protein having an amino acid sequence encoded by the DNA) (2) a method for genetically producing a protein which specifically binds OCIF and has an activity to support and promote differentiation of mouse spleen cells to osteoclasts and maturation thereof by use of the DNA; (3) an agent for treating bone metabolism abnormality comprising the protein; (4) a method for screening a substance which controls expression of human OBM; (5) a method for screening a substance which binds human OBM and inhibits or modifies an effect thereof; (6) a method for screening a receptor which binds human OBM and transmits an effect thereof; and (7) a pharmaceutical composition comprising a substance obtained as a result of these methods for screening.

The present invention relates to DNA which encodes human OBM, a novel human protein, which specifically binds OCIF and has a biological activity to support and promote differentiation and maturation of osteoclasts; a protein having an amino acid sequence encoded by the DNA; a method for genetically producing a protein which

specifically binds OCIF and has an activity to support and promote differentiation and maturation of osteoclasts with the DNA; and an agent for treating bone metabolism abnormality comprising the protein. Furthermore, the present invention also relates to a method for screening a substance which controls expression of human OBM; a method for screening a substance which binds human OBM and inhibits or modifies an effect thereof; a method for screening a receptor which binds human OBM and transmits a biological activity of OBM; a pharmaceutical composition comprising a substance obtained as a result of these methods for screening; an antibody to the human-derived OCIF binding protein; and an agent for preventing and/or treating bone metabolism abnormality using the antibody.

The novel human-derived OCIF binding protein molecule, human OBM, encoded by the DNA of the present invention shows the following physicochemical properties and biological activity. That is, (a) human OBM specifically binds to osteoclastogenesis inhibitory factor (OCIF) (WO 96/26217); (b) human OBM shows a molecular weight of about 40,000 (\pm 5,000) as measured by SDS-PAGE under reducing conditions and shows an apparent molecular weight of about 90,000 to 110,000 when crosslinked with a monomer-type OCIF; and (c) human OBM has a biological activity to support and promote differentiation and maturation of osteoclasts.

The cDNA encoding mouse OBM, mouse-derived OCIF binding protein, useful as a probe for separating and identifying the cDNA which encodes human OBM of the present invention, can be isolated from a cDNA library of a mouse osteoblast-like stromal cell line, ST2. Furthermore, human osteoclastogenesis inhibitory factor (OCIF), required to examine the properties and biological activity of the protein obtained by expressing human OBM cDNA, can be isolated from the culture solution of human fibroblast strain IMR-90 in accordance with the method described in WO 96/26217, or it can be genetically produced with the DNA encoding it. To examine the properties and biological activity of human OBM, recombinant humOCIF, recombinant mouse OCIF, recombinant rat OCIF and the like can also be used. These recombinant OCIFs can be obtained by incorporating the corresponding cDNAs into expression vectors in accordance with a commonly used method, expressing OCIFs in animal or insect cells such as CHO cells, BHK cells and Namalwa cells, and purifying them.

Methods for isolating the human cDNA which encodes the target protein (cDNA cloning) include: (1) a method comprising the steps of purifying the protein, determining a partial amino acid sequence thereof, and isolating the target cDNA by a hybridization with DNA having a nucleotide sequence corresponding to the amino acid sequence as a probe, (2) a method (expression cloning method) comprising the steps of constructing a cDNA library with an expression vector, regardless of whether the amino acid

sequence of the target protein is unknown, introducing them into cells, and screening for the presence and absence of the expression of the target protein so as to isolate the target cDNA; and (3) a method of isolating the cDNA which encodes the target human protein by the hybridization or polymerase chain reaction (PCR) method from cDNA library constructed from human cells or tissue or using cDNA which encodes a protein derived from a mammal other than human and having the same properties and biological activity of the human-derived target protein as a probe, based on an assumption that the cDNA which encodes the non-human protein shares high homology with that which encodes the desired corresponding human protein to be cloned.

Based on an assumption that human OBM cDNA is highly homologous with the above mouse OBM cDNA, human cells or tissue producing human OBM can be identified by Northern hybridization method using the latter (mouse) cDNA as a probe. Human OBM cDNA can be cloned as follows. A human OBM cDNA fragment is obtained through PCR using mouse OBM primers prepared based on the mouse OBM cDNA and the cDNA library of a cell or tissue which produces human OBM (e.g., a human lymph node) as identified above, as primers and a template, respectively. The cDNA library of cells or tissue which produce human OBM as identified above is screened with the human OBM cDNA fragment as a probe, and thus, human OBM cDNA can be obtained. The present invention relates to the obtained DNA that encodes human OBM, a human-derived protein which specifically binds OCIF and has biological activity to support and promote differentiation and maturation of osteoclasts. Since human OBM encoded by the DNA of the present invention is a membrane-bound protein having a transmembrane domain, it can be detected by labeling OCIF and then binding the labeled OCIF to the surface of an animal cell in which the cDNA of the present invention is expressed. In such a case, OCIF can be labeled by a method which is conventionally used for labeling protein such as labeling with a radioisotope and fluorescence labeling.

The molecular weight of the protein expressed by human OBM cDNA of the present invention is determined by gel filtration chromatography, SDS-PAGE and the like. To determine the molecular weight more accurately, SDS-PAGE is preferably used, and human OBM is identified as a protein having a molecular weight of about 40,000 (40,000 \pm 5,000) under reducing conditions.

An example of DNA hybridization under relatively mild conditions in the present invention is that after DNA is transferred to a nylon membrane and fixed in accordance with a commonly used method, the DNA is hybridized with another radiolabeled DNA as a probe in a hybridization buffer at 40° to 70°C for about 2 hours to overnight and then washed with 0.5 X SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 45°C

for 10 minutes. More specifically, after DNA is transferred and fixed to a nylon membrane, which is HYBOND® N (Amersham Co., Ltd.), in accordance with a conventional method, the DNA is hybridized with another ³²P-labeled DNA as a probe in Rapid Hybridization Buffer (Amersham Co., Ltd.) at 65°C for 2 hours, and then washed with the above 0.5X SSC at 45°C for 10 minutes.

As a representative *in vitro* culture system for osteoclastogenesis, a co-culture system of mouse-derived osteoblast-like stromal cell line, ST2, and mouse spleen cells in the presence of the active-form of vitamin D₃ or PTH is well known. For promoting osteoclastogenesis in this *in vitro* culture system, both the interaction between a osteoblast-like stromal cell and a spleen cell through their binding, and the presence of bone resorption factors such as the active-form of vitamin D₃ and PTH are essential. In this *in vitro* culture system, a recombinant COS cell strain, resulting from the expression of the cDNA of the present invention thereon, has obtained an ability to support osteoclast formation from spleen cells, just like the osteoblast-like stromal cell line ST2, while COS-7 cells (a monkey kidney-derived cell line) does not have an ability to support osteoclast formation in the absence of said bone resorption factors. Furthermore, since the cDNA of the present invention encodes a membrane-bound protein, the protein can be expressed as a secretory-type or solubilized-type protein after removing the fragment which encodes the membrane binding domain thereof. It has also been confirmed that osteoclastogenesis was promoted simply by adding the secretory-type human OBM to the above *in vitro* culture system in the absence of said bone resorption factors. From these results, human OBM encoded by the cDNA of the present invention is identified as a factor involved in differentiation and maturation of osteoclasts.

Recombinant human OBM can be produced by inserting the cDNA of the present invention into an expression vector so as to prepare a plasmid for expressing human OBM and then introducing and expressing the plasmid in various cells and strains. COS-7, CHO and Namalwa cells and the like can be used as mammalian host cells suitable for expression, and *E. coli* and the like can be used as bacterial host cells. In those cases, recombinant human OBM can be expressed as a membrane-bound protein by using the full length of DNA or as a secretory-type or solubilized-type protein by removing a region which encodes the membrane binding domain. Thus produced recombinant human OBM can be purified efficiently in combination with conventionally used methods for purifying protein such as affinity chromatography using OCIF-immobilized or a column, ion exchange chromatography, gel filtration chromatography and the like. Thus obtained human OBM of the present invention is useful, due to its activity, as a medicament, e.g., as an agent for treating bone metabolism abnormality such as osteopetrosis or as an experimental and

diagnostic reagent.

The human OBM protein encoded by the cDNA of the present invention enables: (1) screening of a substance which controls expression of human OBM; (2) screening of a substance which specifically binds human OBM and inhibits or modifies the biological activity of human OBM; and (3) screening of a human protein (human OBM receptor) which exists in a precursor cell of human osteoclasts and transmits the biological activity of human OBM, as well as development of antagonist and agonist using this human OBM receptor. In combinatorial chemistry using the above human OBM or human OBM receptor, a peptide library, which is employed for identification of an antagonist or agonist, can be prepared in accordance with the same method using the mouse OBM. After screening the peptide library by said method in which human OBM is used instead of mouse OBM, a specific peptide having very high affinity can be obtained.

Furthermore, for measurement of OBM, a highly useful protein described above, it is necessary to obtain an antibody which specifically recognizes OBM and establish an enzyme immunoassay using it. However, no antibody useful for measurement of OBM has been available. Moreover, an anti-OBM/sOBM antibody which neutralizes the biological activity of OBM or sOBM is assumed to suppress an activity of OBM or sOBM to promote osteoclast formation, and expected to be developed as an agent for treating bone metabolism abnormality. However, such an antibody has not been available.

Under that circumstance, the inventors have made intensive studies and have found antibodies (anti-OBM/sOBM antibodies) which recognize both of the following antigens, a membrane-bound protein (OCIF binding molecule; OBM) which specifically binds osteoclastogenesis inhibitory factor (OCIF) and a soluble-type OBM (sOBM) lacking the membrane binding domain. Therefore, objects of the present invention are to provide: (1) an antibody (anti-OBM/sOBM antibody) which recognizes both of the following antigens, a membrane-bound protein (OBM) which specifically binds to osteoclastogenesis inhibitory factor (OCIF) and soluble OBM (sOBM) lacking the membrane binding domain; (2) a method for production thereof; (3) a method for measuring OBM and the sOBM by use of said antibody; and (4) an agent for preventing and/or treating bone metabolism abnormality which comprises said antibody as an active ingredient.

The present invention relates to: (1) an antibody (anti-OBM/sOBM antibody) which recognizes both of the following antigens, a membrane-bound protein (OCIF binding molecule; OBM) which specifically binds osteoclastogenesis inhibitory factor (OCIF) and a soluble-type OBM (sOBM) lacking the membrane binding domain; (2) a method for production thereof; (3) a method for measuring OBM and the sOBM by use of said antibody; and (4) a pharmaceutical composition comprising said antibody as an active ingredient,

particularly, an agent for preventing and/or treating bone metabolism abnormality.

An antibody of the present invention is an antibody which has an activity to neutralize osteoclastogenesis promoting activity, which is a biological activity that OBM and sOBM have, said antibody has any of the following properties: a) a polyclonal antibody
5 which recognizes both mouse OBM and mouse sOBM antigens (anti-mouse OBM/sOBM polyclonal antibody); b) a polyclonal antibody which recognizes both human OBM and human sOBM antigens (anti-human OBM/sOBM polyclonal antibody); c) a monoclonal antibody which recognizes both mouse OBM and mouse sOBM antigens (anti-mouse OBM/sOBM polyclonal antibody); d) a monoclonal antibody which recognizes both human
10 OBM and human sOBM antigens (anti-human OBM/sOBM polyclonal antibody); and e) an anti-human OBM/sOBM monoclonal antibody which has crossreactivity to both mouse OBM and mouse sOBM antigens.

The polyclonal antibody which recognizes both mouse OBM and mouse sOBM antigens (hereinafter referred to as "anti-mouse OBM/sOBM polyclonal antibody") and the
15 polyclonal antibody which recognizes both human OBM and human sOBM antigens (hereinafter referred to as "anti-human OBM/sOBM polyclonal antibody") can be obtained by the following means. A purified mouse OBM as an antigen for immunization can be obtained in accordance with the above method. That is, natural-type mouse OBM can be obtained by treating a mouse osteoblast-like stromal cell line, ST2, with the active-form of
20 vitamin D₃ and subsequently purifying it from the cell membranes of said cell by means of OCIF immobilized on a column and gel filtration chromatography. Alternatively, after incorporating the above mouse OBM cDNA (SEQ ID NO: 15) or human OBM cDNA (SEQ ID NO: 12) into an expression vector, expressing OBM in an animal or insect cell such as a CHO cell, a BHK cell, Namalwa or a COS-7 cell or *E. coli* and then purifying by the same
25 method as described above, recombinant mouse OBM (SEQ ID NO: 1) or recombinant human OBM (SEQ ID NO: 11) can be obtained, and these may also be used as antigens for immunization. At this time, it takes tremendous effort to highly purify a large quantity of mouse OBM or human OBM, a membrane-bound protein (OBM). On the other hand, it has been confirmed that there is no difference in ability to promote differentiation and maturation
30 of osteoclasts between OBM, a membrane-bound protein, and soluble-type OBM (sOBM), which is a soluble protein obtained by deleting the membrane binding domain of OBM as described above. Accordingly, taking into account that expression and high purification of mouse sOBM and human sOBM are relatively easy, these sOBMs, solubilized proteins, may be used as antigens for immunization. Mouse sOBM (SEQ ID NO: 16) and human sOBM
35 (SEQ ID NO: 17) can be obtained by adding a nucleotide sequence, which encodes a known signal sequence derived from other secretory-type proteins, 5' upstream of the mouse sOBM

cDNA (SEQ ID NO: 18) or the human sOBM cDNA (SEQ ID NO: 19), incorporating the cDNA into an expression vector in accordance with the same gene engineering method as described above, expressing the protein in a variety of animal cells, insect cells or *E. coli* as a host, and then purifying. Thus obtained antigen for immunization is dissolved in a phosphate buffered saline solution (PBS) and, if necessary, mixed with an equal volume of Freund's complete adjuvant and emulsified. Then, an animal is immunized with the emulsion through a few times of subcutaneous administration with a one-week interval between each. The antibody titer is measured. When the value reaches maximum, booster administration is performed. On the 10th day from the booster administration, all the blood was collected. The obtained antiserum is fractionated and precipitated with ammonium sulfate, and the globulin fraction is purified with an anion exchange chromatography or the antiserum is diluted twice with Binding Buffer (Bio-Rad Co., Ltd.) and the diluted antiserum is purified by Protein A or Protein G SEPHAROSE® (Pharmacia Co., Ltd.) column chromatography. Thereby, the desired anti-mouse or anti-human OBM/sOBM polyclonal antibody can be obtained.

The monoclonal antibody of the present invention can be obtained by the following method. That is, as an antigen for immunization required to prepare the monoclonal antibody, a natural-type mouse OBM, recombinant mouse or human OBM, or recombinant mouse or human sOBM can be used, as used in preparation of the above polyclonal antibody. Lymphocytes derived from immunized mammals with each antigen or that obtained by *in vitro* methods are fused with a myeloma cell line, and hybridomas are prepared in accordance with a conventional method. From the culture of this hybridoma, a hybridoma producing an antibody which recognizes each antigen is selected by solid-phase ELISA, using each highly purified antigen. The obtained hybridoma is cloned, and a thus obtained stable antibody-producing hybridoma is cultured. The target antibody can be obtained therefrom. For preparation of the hybridoma, immunizing a mammal, a small animal such as a mouse or rat, is commonly used. To immunize the animal, a method comprising the following steps is conventionally used: diluting the antigen with an appropriate solvent such as physiological saline solution to an appropriate concentration and then administering the solution and, if necessary, co-administering Freund's complete adjuvant, into vein (i.v.) or the abdominal cavity (i.p.), about 3 or 4 times in total with a 1 to 2-week interval between each. The immunized animal is dissected on the 3rd day after the last immunization, and spleen cells are obtained from the isolated spleen and used as immunocytes (immunized cells). Illustrative examples of mouse-derived myeloma for cell fusion with the immunocytes include p3/x63-Ag8, p3-U1, NS-1, MPC-11, SP-2/0, F0, P3x63 Ag8. 653 and S194. Furthermore, illustrative examples of rat-derived cells include cell lines such as R-210. For producing human antibody, human B lymphocyte cells are immunized *in*

vitro and fused with human myeloma cells or a cell line transformed by EB virus. Fusion of an immunized cell with a myeloma cell line is performed according to a known methods such as that of Koehler and Milstein *et al.* (Koehler *et al.*: Nature 256, 495 to 497, 1975), while an electric pulse method using an electric pulse may also be used. Immunized lymphocyte cells and myeloma cell lines are mixed together at a ratio conventionally used and fused in common bovine fetal serum (FCS)-free medium for cell culture in which polyethylene glycol is added. Then, culture is carried out in FCS-containing HAT selective medium so as to select a fused cell (hybridoma). Then, a hybridoma producing an antibody is selected by a commonly used method for detecting antibody such as ELISA, plaque method, ouchterlony method or condensation method. Thereafter, a hybridoma is established. The established hybridoma can be subcultured by a common method for culture and can be stored in a frozen state if necessary. The hybridoma may be cultured in accordance with a conventionally used method or transplanted in the abdominal cavity of the mammal. The antibody can be collected from the resulting culture solution or ascites, respectively. The antibody in the culture solution or ascites can be purified by a commonly used method such as a salting-out method, ion exchange chromatography, gel filtration chromatography, or Protein A or Protein G affinity chromatography. Almost all the monoclonal antibodies obtained by the above-described method using sOBM as an antigen are antibodies which can specifically recognize not only sOBM but also OBM (hereinafter referred to as "anti-OBM/sOBM monoclonal antibody"). These antibodies can be used for measurements of OBM and sOBM. After these antibodies are labeled with a radioisotope or an enzyme and thus employed to measurement systems known such as as radioimmunoassay (RIA) and enzyme immunoassay (EIA), an amount of OBM and sOBM can be measured thereby. By use of these measurement systems, an amount of sOBM in a living sample such as blood or urine can be measured with ease and with high sensitivity. Furthermore, by use of these antibodies, an amount of OBM bound to the surface of a tissue or cell can be measured through a binding assay or the like with ease and with high sensitivity.

When the obtained antibody is used as a medicament for humans, it is desirable, in consideration of a problem of antigenicity, that a human-type anti-human OBM/sOBM antibody is prepared. The human-type anti-human OBM/sOBM antibody can be prepared by the following methods. That is, (1) human lymphocyte cells extracted from human peripheral blood or the spleen are sensitized *in vitro* with human OBM or human sOBM as an antigen in the presence of IL-4, and then the sensitized human lymphocyte cells are fused with K₆H₆/B₅ (ATCC CRL1823), which is a hetero-hybridoma of mouse and human, thereby, a hybridoma producing the desired antibody is screened. An antibody produced from the obtained hybridoma is a human-type anti-human OBM/sOBM monoclonal antibody. Among these

antibodies, an antibody which neutralizes the activity of human OBM/sOBM is selected. However, it is usually difficult to obtain an antibody having high affinity for an antigen through such a method of sensitizing human lymphocyte cells *in vitro*. Therefore, for obtaining a monoclonal antibody having high affinity for human OBM and sOBM, it is necessary to modify an anti-human OBM/sOBM monoclonal antibody with low affinity as described above to be that with high affinity. A random mutation is introduced into a CDR region (CDR-3 in particular) of said human-type anti-human OBM/sOBM monoclonal antibody with low affinity which a neutralizing antibody obtained as described above. This is expressed with phage. Phage which strongly bind human OBM/sOBM as the antigen are selected by phage display method using a plate in which human OBM/sOBM is immobilized. The phage is allowed to proliferate in *E. coli*, and the deduced amino acid sequence of the CDR having high affinity is determined based on the nucleotide sequence thereof. The obtained gene which encodes the human-type anti-human OBM/sOBM monoclonal antibody is incorporated and expressed in a conventionally used expression vector for mammalian cells, and then human-type anti-human OBM/sOBM monoclonal antibodies can be obtained. Among them, the desired human-type anti-human OBM/sOBM monoclonal antibody which neutralizes the biological activity of human OBM/sOBM and has high affinity thereto can be selected. Furthermore, (2) using a Balb/c mouse, an anti-human OBM/sOBM mouse monoclonal antibody is prepared according to a conventionally used method (Koehler *et al.*, Nature 256, 495 to 497, 1975) as in the present invention, and a monoclonal antibody which neutralizes the biological activity of human OBM/sOBM and has high affinity thereto is selected. By CDR grafting method (Winter and Milstein: Nature 349, 293 to 299, 1991), that is a method in which a CDR region (CDR-1, 2 and 3) of the anti-human OBM/sOBM mouse monoclonal antibody with high affinity are transplanted into the CDR regions of human IgG, a humanized antibody can be obtained. Moreover, (3) human peripheral blood lymphocyte cells are transplanted into a severe combined immune deficiency (SCID) mouse. Thus transplanted SCID mouse produces a human antibody (Mosier D. E. *et al.*: Nature 335, 256 to 259, 1988; Duchosal M. A. *et al.*: Nature 355, 258 to 262, 1992). The cells are sensitized with human OBM or sOBM as an antigen and screened. Thereafter, a lymphocyte cell which produces a human-type monoclonal antibody specific to human OBM/sOBM can be extracted from the mouse. Then, as in the case of the above method for preparing a human-type antibody (1), the obtained lymphocyte cells are fused with K₆H₆/B₅ (ATCC CRL1823), a hetero hybridoma of mouse and human, and then the obtained hybridomas are screened. Then, a hybridoma which produces the target human-type monoclonal antibody can be obtained. By culturing the thus obtained hybridoma, the target human-type monoclonal antibody can be produced in large quantities. After purifying them in the same manner as

described above, large amounts of pure products thereof can be obtained. Furthermore, a recombinant human-type monoclonal antibody can be produced in large quantities by constructing a cDNA library from said hybridoma which produces the target human-type monoclonal antibody, cloning the cDNA which encodes the target human-type monoclonal antibody, incorporating said cDNA into an appropriate expression vector by gene engineering, and expressing the antibody in a variety of animal cells, insect cells or *E. coli* as a host. After purification of the antibody from said culture according to the method as described above, a large amount of pure human-type monoclonal antibody can be obtained.

Among the anti-OBM/sOBM monoclonal antibodies obtained by the above method, moreover, an antibody which neutralizes the biological activity of OBM/sOBM can be obtained. These antibodies which neutralize the biological activity of OBM/sOBM are expected as medicaments, particularly agents for preventing and/or treating bone metabolism abnormality, since they can inhibit the biological action (an activity to promote osteoclast formation) of OBM/sOBM in a living body. The activity of the anti-OBM/sOBM antibody to neutralize the biological activity of OBM or sOBM can be determined as an activity to inhibit osteoclast formation in an *in vitro* system for examining osteoclast formation. As *in vitro* assay systems, the following three methods can be used. That is, *in vitro* culture systems for examining osteoclastogenesis include: (1) a co-culture system of a mouse osteoblast-like stromal cell line, ST2, and mouse spleen cells in the presence of the active-form of vitamin D₃ and dexamethasone; (2) a co-culture system in which OBM is expressed on a monkey kidney cell line, COS-7, and fixed with formaldehyde, and then mouse spleen cells are cultured on the cells in the presence of M-CSF; and (3) a system of culturing mouse spleen cell in the presence of recombinant sOBM and M-CSF; however, other systems can be also used. When an anti-OBM/sOBM antibody is added to such a culture system in various concentration and its effect on osteoclastogenesis is examined, an activity of the anti-OBM/sOBM antibody to inhibit osteoclastogenesis can be measured. Also, the activity of the anti-OBM/sOBM antibody to inhibit osteoclastogenesis can be determined as an activity to suppress bone resorption *in vivo* using an experimental animal. That is, there is an animal model, an ovariectomized mouse, in which osteoclastogenesis is increased. An anti-OBM/sOBM antibody is administered to such a kind of experimental animal, and an activity to inhibit bone resorption (an activity to reinforce bone mineral density) is measured. Thereby, an activity of the anti-OBM/sOBM antibody to inhibit osteoclastogenesis can be determined.

The obtained antibody, which neutralizes the biological activity of OBM/sOBM is useful as a medicament, particularly as a pharmaceutical composition for preventing and/or treating bone metabolism abnormality, or as an antibody used in immunological diagnosis of

such a disease. The antibody of the present invention can be prepared in a formulation, and administered orally or parenterally. A formulation comprising the antibody of the present invention is administered safely to human or animal as a pharmaceutical composition comprising the antibody which recognizes OBM and/or sOBM as an active ingredient.

5 Illustrative examples of the formulation of the pharmaceutical composition include injectable solutions such as drip, suppository, nasogastric agent, sublingual agent and transdermal agent. Since the monoclonal antibody has a high molecular weight, its adsorption to glass containers such as vial and syringe tube is significant. Furthermore, the antibody is unstable and easily inactivated due to various physicochemical factors such as heat, pH, and humidity.

10 Thus, to stably formulate the antibody, stabilizer, pH adjuster, buffer, solubilizing agent, surfactant and the like are added thereto. Illustrative examples of the stabilizer include amino acids such as glycine and alanine, saccharides such as dextran 40 and mannose, and sugar alcohols such as sorbitol, mannitol and xylitol. These may be used in combinations of two or more. These stabilizers are preferably added in an amount which is 0.01 to 100 times, particularly 0.1 to 10 times, as much as the weight of the antibody. By addition of these stabilizers, the storage stability of liquid formulation or freeze-dried formulation can be improved. Illustrative examples of buffer include phosphate buffer and citric buffer. The buffer adjusts the pH of an aqueous formulation or a reconstituted solution of freeze-dried formulation, and thereby contributes to the stability and solubility of the antibody therein.

20 The amount of the buffer is preferably, for example, 1 to 10 mM in an aqueous formulation or a reconstituted solution of freeze-dried formulation. The surfactant is preferably polysorbate 20, PLURONIC® (BASF Co., Co.) F-68 and polyethylene glycol, particularly preferably polysorbate 20. These may be used in combinations of two or more. A protein having high molecular weight like an antibody is liable to adsorb to glass or resin, which a container is made of. However, by addition of a surfactant adsorption of the antibody to a container in an aqueous formulation or a reconstituted solution of freeze-dried formulation can be prevented. The surfactant is preferably added in an amount of 0.001 to 1.0% of the weight of an aqueous formulation or a reconstituted solution of freeze-dried formulation. The formulation comprising the antibody of the present invention can be prepared by addition of the stabilizer, buffer and adsorption-preventing agent as described above. Particularly, when it is used as an injectable formulation for medical applications or treating animals, acceptable osmotic pressure ratio is preferably 1 or 2. The osmotic pressure ratio can be adjusted by increasing or decreasing sodium chloride in formulation. The content of the antibody in the formulation can be adjusted appropriately, dependent on the disease to be treated with said formulation, administration route and the like. The dose of the human-type antibody administered to humans depends on the affinity of the antibody to human OBM/sOBM, that

is, the dissociation constant (K_d value) of the antibody to human OBM/sOBM. The higher the affinity is (or the lower the K_d value is), the smaller the dose that is required to exhibit medicinal benefits. Furthermore, since the half-life time of human-type antibodies in human blood is about 20 days, the human-type antibody can be administered to humans in a dose of about 0.1 to 100 mg/kg at least once within 1 to 30 days, for example.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the results of SDS-PAGE of mouse OBM protein of Example 3 of the present invention, wherein:

(A): Lane 1: molecular weight marker,

Lane 2: partially purified fraction eluted with Gly-HCl (pH 2.0), which was derived from ST2 cells cultured in the presence of the active-form of vitamin D_3 and dexamethasone,

Lane 3: partially purified fraction eluted with Gly-HCl (pH 2.0), which was derived from ST2 cells cultured in the absence of the active-form of vitamin D_3 and dexamethasone,

(B): Lane 1: molecular weight marker,

Lane 2: mouse OBM protein (Example 3) of the present invention purified with reversed phase high performance liquid chromatography.

Fig. 2 shows the results of binding experiment of the ^{125}I -labeled OCIF to an osteoblast-like stromal cell, ST2, in Example 4.

Fig. 3 shows the osteoclastogenesis-supporting activity of osteoblast-like stromal cell line, ST2, with different passage numbers, in Example 5(1), wherein:

1: osteoclastogenesis-supporting activity of ST2 cells with a passage number of around 10's,

2: osteoclastogenesis-supporting activity of ST2 cells with a passage number of around 40's.

Fig. 4 shows a change in expression of the protein of the present invention on an osteoblast-like stromal cell membrane, said cells were cultured in the presence of an active-form of vitamin D_3 and dexamethasone, with passage of time, in Example 5(2).

Fig. 5 shows a change in osteoclastogenesis in the co-culture system, with passage of time, of Example 5(2).

Fig. 6 shows osteoclastogenesis-inhibiting effects when OCIF was treated only during various culturing periods in the co-culture period of Example 5(3).

Fig. 7 shows the results of crosslinking experiment of the ^{125}I -labeled OCIF with the protein of the present invention, in Example 6, wherein:

Lane 1: ^{125}I -labeled OCIF-CDD1,

Lane 2: sample resulting from crosslinking of ^{125}I -labeled OCIF-CDD1 with an ST2 cell line,

Lane 3: sample resulting from crosslinking an ST2 cell in the presence of a 400-fold higher concentration of unlabeled OCIF than that of ^{125}I -OCIF.

Fig. 8 shows the results of SDS-PAGE in Example 9, wherein:

Lane 1: precipitate resulting from immuno precipitation of the protein of COS-7 cells transfected with pOBM291 without OCIF,

Lane 2: precipitate resulting from immuno precipitation of the protein of COS-7 cells transfected with pOBM291 with OCIF.

Fig. 9 shows the results of binding experiment of ^{125}I -labeled OCIF to COS-7 cells transfected with pOBM291 in Example 10, wherein:

Lanes 1 and 2: amount of ^{125}I -labeled OCIF bound to COS-7 cells transfected with pOBM291,

Lanes 3 and 4: amount of ^{125}I -labeled OCIF bound to COS-7 cells transfected with pOBM291 in the presence of a 400-fold higher concentration of unlabeled OCIF than that of ^{125}I -OCIF.

Fig. 10 shows the results of crosslinking experiment using ^{125}I -labeled OCIF in Example 11, wherein:

Lane 1: ^{125}I -labeled OCIF,

Lane 2: sample resulting from crosslinking of ^{125}I -labeled OCIF with COS-7 cells transfected with pOBM291,

Lane 3: sample resulting from crosslinking of ^{125}I -labeled OCIF with COS-7 cells transfected with pOBM291 in the presence of a 400-fold higher concentration of unlabeled OCIF than that of ^{125}I -OCIF.

Fig. 11 shows the results of Northern blot in Example 12, wherein:

Lane 1: RNA derived from ST2 cells cultured in the absence of vitamin D and dexamethasone,

Lane 2: RNA derived from ST2 cells cultured in the presence of vitamin D and dexamethasone.

Fig. 12 shows OCIF binding ability of a protein in the conditioned medium when the concentration of OCIF was varied in Example 14-(2), wherein:

○: pCEP4,

●: pCEP sOBM.

Fig. 13 shows the OCIF binding ability of the protein in the conditioned medium when the amount of the conditioned medium was varied in Example 14-(2), wherein:

○: pCEP4,

●: pCEP sOBM.

Fig. 14 shows the results of SDS-PAGE of a fusion protein of thioredoxin and mouse OBM expressed in *E. coli*, in Example 15-(2), wherein:

Lane 1: molecular weight marker,

Lane 2: soluble protein fraction derived from GI724/pTrxFus,

Lane 3: soluble protein fraction derived from GI724/pTrxOBM25.

Fig. 15 shows OCIF binding abilities when the amount of the soluble protein fractions were varied in Example 15-(3), wherein:

□: GI724/pTrxFus,

5 ○: GI724/pTrxOBM25.

Fig. 16 shows the OCIF binding abilities of the soluble protein fractions (1%) when the concentration of OCIF was varied in Example 15-(3), wherein:

□: GI724/pTrxFus,

○: GI724/pTrxOBM25.

10 Fig. 17 shows the results of inhibition of the specific binding of the mouse protein obtained by expressing the mouse OBM cDNA of the present invention and purifying (mouse OBM) and the purified natural-type OCIF binding protein to OCIF, by an anti-mouse OBM rabbit antibody, wherein:

1: purified recombinant OBM treated with an antibody + ^{125}I -OCIF,

15 2: the purified natural-type protein treated with an antibody + ^{125}I -OCIF,

3: purified recombinant OBM untreated with an antibody + ^{125}I -OCIF,

4: the purified natural-type protein untreated with an antibody + ^{125}I -OCIF,

5: 3 + unlabeled OCIF (400-fold higher concentration than that of ^{125}I -OCIF),

6: 4 + unlabeled OCIF (400-fold higher concentration than that of ^{125}I -OCIF).

20 Fig. 18 shows the results of SDS-PAGE of human OBM protein expressed by the cDNA of the present invention, wherein:

Lane 1: molecular weight marker,

Lane 2: precipitate resulting from immuno precipitation of the protein derived from COS-7 cells transfected with an expression vector (phOBM) containing the cDNA of the present invention by an anti-OCIF rabbit polyclonal antibody without OCIF,

25 Lane 3: precipitate resulting from immuno precipitation of the protein derived from COS-7 cells transfected with an expression vector (phOBM) containing the cDNA of the present invention by an anti-OCIF rabbit polyclonal antibody with OCIF.

Fig. 19 shows the results of a binding experiment of OCIF to COS-7 cells transfected with an expression vector (phOBM) containing the cDNA of the present invention, wherein:

Lane 1: COS-7 cells transfected with phOBM + ^{125}I -OCIF,

Lane 2: COS-7 cells transfected with phOBM + ^{125}I -OCIF + a 400-fold higher concentration of unlabeled OCIF than that of ^{125}I -OCIF.

35 Fig. 20 shows the results of crosslinking experiment of human OBM protein encoded by the cDNA of the present invention with ^{125}I -OCIF (monomer type), wherein:

Lane 1: ^{125}I -OCIF,

Lane 2: sample resulting from crosslinking of ^{125}I -OCIF with a protein on the membrane of COS-7 cells transfected with phOBM,

Lane 3: sample resulting from crosslinking of ^{125}I -OCIF with a protein on the membrane of COS-7 cells transfected with phOBM in the presence of a 400-fold higher concentration of unlabeled OCIF than that of ^{125}I -OCIF.

Fig. 21 shows the OCIF binding ability of a protein (secretory-type human OBM) in the conditioned medium when the concentration of OCIF was varied in Example 24-(2), wherein:

- : conditioned medium of 293-EBNA cell transfected with pCEP4 vector not containing cDNA which encodes the secretory-type human OBM,
- : conditioned medium of 293-EBNA cell transfected with pCEPshOBM expression vector containing cDNA which encodes the secretory-type human OBM.

Fig. 22 shows the OCIF binding ability of the protein (secretory-type human OBM) in the conditioned medium when the amount of the conditioned medium to be added was varied while the concentration of OCIF was kept constant, in Example 24-(2), wherein:

- : conditioned medium of 293-EBNA cells transfected with pCEP4 vector not containing cDNA which encodes the secretory-type human OBM,
- : conditioned medium of 293-EBNA cells transfected with pCEPshOBM expression vector containing cDNA which encodes the secretory-type human OBM.

Fig. 23 shows the results of SDS-PAGE of a fusion protein of thioredoxin and human OBM, expressed in *E. coli*, wherein:

Lane 1: molecular weight marker,

Lane 2: soluble protein fraction derived from *E. coli* GI724/pTrxFus,

Lane 3: soluble protein fraction derived from *E. coli* GI724/pTrxOBM.

Fig. 24 shows the ability of a fusion protein to bind OCIF when the amount of soluble protein fraction containing the fused protein of thioredoxin and human OBM expressed in *E. coli* was varied, in Example 25-(3), wherein:

- : soluble protein fraction derived from *E. coli* GI724/pTrxFus,
- : soluble protein fraction derived from *E. coli* GI724/pTrxshOBM.

Fig. 25 shows the ability of the fusion protein of thioredoxin and human OBM in a soluble protein fraction of *E. coli* to bind OCIF when the concentration of OCIF was varied, in Example 25-(3), wherein:

- : soluble protein fraction derived from *E. coli* GI724/pTrxFus
- : soluble protein fraction derived from *E. coli* GI724/pTrxshOBM.

Fig. 26 shows the results of measurement of human OBM and human sOBM by

sandwich ELISA using an anti-human OBM/sOBM rabbit polyclonal antibody of the present invention, wherein:

□: human OBM,

●: human sOBM.

5 Fig. 27 shows the results of measurement of human OBM and human sOBM by sandwich ELISA using an anti-human OBM/sOBM monoclonal antibody of the present invention, wherein:

□: human OBM,

●: human sOBM.

10 Fig. 28 shows the results of measurement of mouse OBM and mouse sOBM by sandwich ELISA using an anti-human OBM/sOBM monoclonal antibody of the present invention, said antibody has cross-reactivity to both mouse OBM and mouse sOBM, wherein:

□: mouse OBM,

●: mouse sOBM.

15 Fig. 29 shows an activity of a fusion protein of thioredoxin and mouse OBM to promote the formation of human osteoclast-like cells

Fig. 30 shows suppression of vitamin D₃-stimulated bone resorption by an anti-OBM/sOBM antibody.

20 Fig. 31 shows suppression of prostaglandin E₂ (PGE₂)-stimulated bone resorption by an anti-OBM/sOBM antibody.

Fig. 32 shows suppression of parathyroid hormone (PTH)-stimulated bone resorption by an anti-OBM/sOBM antibody.

Fig. 33 shows suppression of interleukin 1 α (IL-1)-stimulated bone resorption by an anti-OBM/sOBM antibody.

25

BEST MODE FOR PRACTICING THE INVENTION

[Examples]

30 The present invention is explained in more detail with reference to the following Examples. However, these Examples are only exemplary and shall not limit the present invention thereto in any way.

[Example 1]

Production of the Protein of the Present Invention

(1) Large Scale Culture of ST2 Cells

35 Mouse osteoblast like stromal cell line, ST2, (Riken Cell Bank, RCB0224) was cultured with α -MEM medium containing 10% bovine fetal serum. After cultured to become confluent in a 225-cm² T flask for adherent cells, ST2 cells were treated with trypsin,

stripped from the T flask, washed, and then transferred to five of 225-cm² T flask. After addition of 60 ml of α -MEM medium containing 10⁻⁸ M of the active-form of vitamin D₃ (calcitriol), 10⁻⁷ M dexamethasone and bovine fetal serum, the resulting cells were cultured in a CO₂ incubator for 7 to 10 days. The cultured ST2 cells were recovered using a cell scraper and stored at -80°C until use.

(2) Preparation of Membrane Fraction and Solubilization of Membrane-Bound Protein

To ST2 cells (amount: about 12 ml) described in Example 1-(1) which were cultured with 80 of 225-cm² T flasks, a 3-fold volume (36 ml) of 10 mM Tris-hydrochloric acid buffer (pH 7.2) containing protease inhibitors (2 mM APMSFP, 2 mM EDTA, 2 mM o—phenanthroline, 1 mM leupeptin, 1 μ g/ml pepstatin A and 100 units/ml aprotinin) were added. After these cells were vigorously agitated by use of a vortex mixer for 30 seconds, they were left to stand on ice for 10 minutes. Using a homogenizer (Dounce Tissue Grinder, A syringe, Wheaton Scientific Co., Ltd.), these cells were crushed. To the crushed cell solution, an equal volume (48 ml) of 10 mM Tris-hydrochloric acid buffer (pH 7.2) containing the above protease inhibitors, 0.5 M sucrose, 0.1 M potassium chloride, 10 mM magnesium chloride and 2 mM calcium chloride was added. The obtained mixture was agitated and then centrifuged at 600 x g at 4°C for 10 minutes. Through this centrifugation, cell nuclei and uncrushed cells were separated as precipitated fractions. A supernatant obtained after centrifugation was further centrifuged at 150,000 x g at 4°C for 90 minutes, and membrane fractions of the ST2 cells were obtained as precipitated fractions. To the membrane fractions, 8 ml of 10 mM Tris-hydrochloric acid buffer (pH 7.2) containing the above protease inhibitors, 150 mM of sodium chloride, and 0.1 M sucrose was added, and then 200 μ l of 20% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, aigma Co., Ltd.) was added. The mixture was agitated at 4°C for 2 hours. This solution was centrifuged at 150,000 x g at 4°C for 60 minutes, and the resulting supernatant was obtained as a solubilized membrane fraction.

[Example 2]

Purification of the Protein of the Present Invention

(1) Preparation of OCIF-Immobilized Affinity Column

Isopropanol in a HITRAP® NHS-activated column (1 ml, Pharmacia Co., Ltd.) was substituted with 1 mM hydrochloric acid, and 1 ml of 0.2 M NaHCO₃/0.5 M NaCl (pH 8.3) solution containing 13.0 mg of recombinant OCIF prepared in accordance with a method described in WO 96/26217 was added to the column using a syringe (5 ml, Terumo Corporation). After the column was allowed to undergo a coupling reaction at room temperature for 30 minutes, 3 ml of 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 3 ml of 0.1 M acetic acid/0.5 M NaCl (pH 4.0) were loaded on the column alternately three times each in

total so as to inactivate excessive activated groups. Then, the mobile phase of the column was substituted again with 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and then left to stand at room temperature for 1 hour. Thereafter, the resulting column was washed twice with 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH 4.0) and then the mobile phase was substituted with 50 mM Tris/1M NaCl/0.1% CAHPS buffer (pH 7.5).

(2) Purification of the Protein of the Present Invention by an OCIF-Immobilized Affinity Column

Purification of OCIF binding protein was carried out at 4°C unless otherwise stated. The above OCIF-immobilized affinity column was equilibrated with 10 mM Tris-hydrochloric acid buffer (pH 7.2) containing the protease inhibitors described in Example 1-(2), 0.15 M sodium chloride and 0.5% CHAPS. To this column, about 8 ml of the solubilized membrane fraction described in Example 1-(2) was applied at a flow rate of 0.01 ml/min. The column was washed with the above 10 mM Tris-hydrochloric acid buffer (pH 7.2) containing the protease inhibitors, 0.15 M sodium chloride and 0.5% CHAPS at a flow rate of 0.5 ml/min for 100 minutes. Then, the proteins were eluted from the column with 0.1 M glycine-hydrochloric acid buffer (pH 3.3) containing the above protease inhibitors, 0.2 M sodium chloride and 0.5% CHAPS at a flow rate of 0.1 ml/min for 50 minutes. Similarly, a 0.1 M sodium citrate buffer (pH 2.0) containing said protease inhibitors, 0.2 M sodium chloride and 0.5% CHAPS was fed to the column at a flow rate of 0.1 ml/min for 50 minutes so as to elute proteins adsorbed to the column. The eluates were fractionated as 0.5 ml/fraction each. The fractions were immediately neutralized by addition of a 2M Tris solution. The fractions (the volume of the eluate was 1.0 to 5.0 ml) eluted with the buffer were concentrated to 50 to 100 µl using CENTRICON®-10 (Amersham Co., Ltd.). Aliquots of the concentrated fractions were subfractionated, and after addition of OCIF to the aliquots, they were immunoprecipitated with an anti-OCIF polyclonal antibody. After the precipitated fractions were treated with SDS, they were subjected to SDS-PAGE, and then a fraction (Fr. Nos. 3-10) showing a band of the protein having an activity to specifically bind OCIF was identified as the protein fraction of the present invention.

(3) Purification of the Protein of the Present Invention by Gel Filtration

The OCIF binding protein eluted with 0.1 M glycine-hydrochloric acid buffer (pH 3.3) and subsequently 0.1 M sodium citrate buffer (pH 2.0) after purification and concentration in accordance with the method described in Example 2-(2) was subjected to a SUPEROSE® 12 HR10/30 column (Pharmacia Co., Ltd., 1.0 X 30 cm) equilibrated with 10 mM Tris-HCl, 0.5 M NaCl and 0.5% CHAPS (pH 7.0) and developed using the above equilibration buffer as a mobile phase at a flow rate of 0.5 ml/min, and then fractions of 0.5 ml were collected. The fraction containing the protein of the present invention (Fr. Nos. 27-

32) was identified and concentrated by means of CENTRICON®-10 (Amersham Co., Ltd.) in the same manner as described above.

(4) Purification by Reversed Phase High Performance Liquid Chromatography

OCIF binding protein purified by the above gel filtration was added to a C₄ column (2.1 X 250 mm, Vydac, USA) equilibrated with 0.1% trifluoroacetic acid (TFA) and 30% acetonitrile. Elution was carried out at a flow rate of 0.2 ml/min with the gradient of acetonitrile concentration of from 30% to 55% for 50 minutes and then of from 55% to 80% for another 10 minutes. The peaks of eluted proteins were detected at 215 nm. The eluted protein of each peak was fractionated, and the peak of the protein of the present invention was identified. Thus, a highly purified protein of the present invention was obtained.

[Example 3]

SDS-PAGE of the Purified Protein of the Present Invention

First, a solubilized membrane fraction prepared from ST2 cells which were cultured in the presence or absence of the active-form of vitamin D₃ was purified with the OCIF-immobilized affinity column as described above, and the purified samples were subjected to SDS-PAGE. As shown in Fig. 1(A), it was revealed that a major protein band of about 30,000 to 40,000 was detected only in the purified sample obtained from the ST2 cells cultured in the presence of the active-form of vitamin D₃, and that a protein which specifically binds OCIF, i.e., the protein of the present invention, is selectively concentrated and purified with the OCIF-immobilized affinity column. However, in addition to the protein of the present invention, some other bands of proteins which were nonspecifically bound to the carriers, spacers or the like of the OCIF-immobilized column were also detected in both purified samples. These proteins other than the protein of the present invention were removed by gel filtration and C₄ reversed phase chromatography as described above. The SDS-PAGE of the obtained highly purified protein of the present invention is shown in Fig. 1(B). The highly purified protein of the present invention was electrophoretically homogeneous, and the molecular weight thereof was about 30,000 to 40,000.

[Example 4]

Examining the Binding of OCIF to Osteoblasts

(1) Preparation of ¹²⁵I-Labeled OCIF

OCIF was ¹²⁵I-labeled by Iodogen method. More specifically, 20 µl of 2.5 mg/ml Iodogen-chloroform solution was transferred to a 1.5 ml Eppendorf tube, and chloroform was evaporated at 40°C so as to prepare an Iodogen-coated tube. After the tube was washed three times with 400 µl of 0.5 M sodium phosphate buffer (Na-Pi, pH 7.0), 5 µl of 0.5 M Na-Pi (pH 7.0) was added thereto. Immediately after 1.3 µl (18.5 MBq) of Na-¹²⁵I solution (Amersham Co., Ltd., NEZ-033H20) was added to the tube, 10 µl of 1 mg/ml rOCIF

solution (monomer type or dimer type) was added. The obtained solution was agitated with a vortex mixer, and then left to stand at room temperature for 30 seconds. The solution was transferred to a tube containing 80 µl of 10 mg/ml potassium iodide and 0.5 M Na-Pi solution (pH 7.0), and 5 µl of phosphate buffered saline solution containing 5% bovine serum albumin, and then agitated. This solution was applied to a spin column (1 ml, G-25 fine, Pharmacia Co., Ltd.) equilibrated with a phosphate buffered saline solution containing 0.25% bovine serum albumin and centrifuged at 2,000 rpm for 5 minutes. After adding 400 µl of phosphate buffered saline solution containing 0.25% bovine serum albumin to the fraction eluted from the column and subsequently mixed, 2 µl aliquots were collected, and the radioactivity thereof was measured with a gamma counter. The radiochemical purity of the thus prepared ¹²⁵I-labeled OCIF solution was determined by measuring the radioactivity of a fraction precipitated by 10% TCA. Furthermore, the biological activity as OCIF the ¹²⁵I-labeled OCIF solution was determined in accordance with a method described in WO 96/26217. Moreover, the concentration of ¹²⁵I-labeled OCIF was measured by ELISA in the following manner.

(2) Measurement of Concentration of ¹²⁵I-Labeled OCIF by ELISA

100 µl of 50 mM NaHCO₃ (pH 9.6) in which 2 µg/ml of anti-OCIF rabbit polyclonal antibody described in WO 96/26217 was dissolved was added to each well of 96-well immunoplate (MaxiSorp, Nunc Co., Ltd.) and left to stand at 4°C overnight. After this solution was removed, 300 µl of BLOCKACE (Snow Brand Milk Products Co., Ltd.)/phosphate buffered saline solution (25/75) was added to each well and then left to stand at room temperature for 2 hours. After this solution was removed, each well was washed three times with phosphate buffered saline solution (P-PBS) containing 0.01% polysorbate 80. Thereafter, 300 µl of BLOCKACE/phosphate buffered saline solution (25/75) containing ¹²⁵I-labeled OCIF sample or standard OCIF was added to each well and left to stand at room temperature for 2 hours. After this solution was removed, each well was washed six times with 200 µl of P-PBS. Then, 100 µl of BLOCKACE (Snow Brand Milk Products Co., Ltd.)/phosphate buffered saline solution (25/75) containing peroxidase-labeled anti-OCIF rabbit polyclonal antibody was added to each well and left to stand at room temperature for 2 hours. After this solution was removed, each well was washed six times with 200 µl of P-PBS. Then, 100 µl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well and then left to stand at room temperature for 2 to 3 minutes. Thereafter, 100 µl of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at 490 nm was measured with a microplate reader. The concentration of the ¹²⁵I-labeled OCIF was calculated from a calibration curve made by using

standard OCIF.

(3) Examining the Binding of OCIF to Osteoblasts or Pancreas Cells

Mouse osteoblast-like stromal cell line, ST2, or mouse pancreas cells were suspended in α -MEM medium containing 10% bovine fetal serum (FBS) with or without 10^{-8} M of the active-form of vitamin D₃ (calcitriol) and 10^{-7} M dexamethasone at a concentration of 4×10^4 cell/ml and 2×10^6 cell/ml, respectively. 1 ml of this medium was seeded in a 24 well microplate. After the cells were cultured in a CO₂ incubator for 4 days and washed with α -MEM medium, 200 μ l of medium for the binding experiment (α -MEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer and 0.2% NaN₃) further containing 20 ng/ml of the above ¹²⁵I-labeled OCIF (monomer type or dimer type) was added to each well. Furthermore, 200 μ g/ml of the medium for the binding experiment containing 8 μ g/ml rOCIF (400-fold higher concentration) was added to other wells which were subjected to measurements of nonspecific binding. After the cells were cultured in a CO₂ incubator for 1 hour, they were washed three times with 1 ml of phosphate buffered saline solution. Since pancreas cells are floating cells, cells in each well were washed in the 24-well plate with centrifugation. After washing, 500 μ l of 0.1 N NaOH solution was added to each well and left to stand at room temperature for 10 minutes. Thereby, the cells were washed, and the amount of RI bound to the cells was measured with a gamma counter.

The ¹²⁵I-labeled OCIF did not bind the cultured pancreas cells, but specifically bound only osteoblast like stromal cells cultured in the presence of the active-form of vitamin D₃. Thereby, it was revealed that the protein of the present invention was a membrane-bound protein induced on the cell surface of osteoblast like stromal cells with the active-form of vitamin D₃ and dexamethasone.

[Example 5]

Biological Activity of the Protein of the Present Invention

(1) Ability of Osteoblast Like Stromal Cells to Support Osteoclast Formation

The ability of osteoblasts to support osteoclast formation was examined by measuring tartaric acid resistant acid phosphatase activity (TRAP activity) of the formed osteoclasts. More specifically, mouse osteoblast-like stromal cell line, ST2, (5×10^3 cells/100 μ l/well) (2×10^5 cells/100 μ l/well) and pancreas cells derived from a ddy mouse (8 to 12 weeks old) were suspended in α -MEM medium containing 10% bovine fetal serum, 10^{-8} M of the active-form of vitamin D₃ and 10^{-7} M dexamethasone and seeded in a 96-well plate. After the cells were cultured in a CO₂ incubator for one week, each well was washed with phosphate buffered saline solution. Then, 100 μ l of ethanol/acetone (1:1) was further added to the wells and fixed at room temperature for 1 minute. Then, 100 μ l of 50 mM citric

acid buffer (pH 4.5) containing 5.5 mM p-nitrophenol phosphate and 10 mM sodium tartrate was added to each well and then allowed to react at room temperature for 15 minutes. After the reaction, 0.1 N NaOH solution was added to each well, and the absorbance at 405 nm was measured with a microplate reader. Fig. 3 shows the results of examining the abilities of ST2 cells to support osteoclast formation, wherein the passage number of said cells were around 10's or around 40's (after purchased from Riken Cell Bank). From these results, it was revealed that ST2 cells of a high passage number had a high ability to support osteoclast formation.

(2) Changes with the Passage of Time in Expression of the Protein of the Present Invention on the Menbrane of Osteoblast Like Stromal Cells Cultured in the Presence of the Active-Form of Vitamin D₃ and Dexamethasone and Those in Osteoclast Formation in a Co-Culture System

Osteoblast-like stromal cell line, ST2, was cultured in the presence of the active-form of vitamin D₃ and dexamethasone for 7 days in the same manner as in Example 4-(3). OCIF-binding experiment was conducted using ¹²⁵I-labeled OCIF (monomer type) described in Example 4-(1). Nonspecific binding was measured by competing the ¹²⁵I-labeled OCIF with a 400-fold higher concentration of unlabeled OCIF in binding to ST2 cells. As a result, the amount of specific binding of the ¹²⁵I-labeled OCIF was increased, due to the active-form of vitamin D₃ and dexamethasone, with an increase in culturing days. That is, as shown in Figures 4 and 5, the protein of the present invention was expressed on the cell surface of ST2 cells due to the active-form of vitamin D₃ with an increase in culturing days, and its expression reached maximum on the fourth day of culture. On the other hand, osteoclast-like cells were formed after co-culture of mouse spleen cells and ST2 cells in the presence of the active-form of vitamin D₃. TRAP (a marker enzyme for osteoclasts)-positive mononuclear osteoclast-like cells were formed on the third or fourth day of culture, and further, differentiated and matured TRAP-positive multinuclear cells were formed on the fifth or sixth day of culture. It was found that change with the passage of time in expression of the protein of the present invention and in osteoclast formation corresponded well with each other.

(3) Effect of Inhibiting Osteoclast Formation When OCIF Was Treated Only During a Restricted Period of Co-Culture

To further clarify that the protein of the present invention was a factor involved in osteoclast formation, cells cultured during various periods (two days each, except for the fifth day) were treated with 100 ng/ml of OCIF in the above 6-day co-culture described in Example 5-(2). As a result shown in Fig. 6, in the case where OCIF was added during the 48th to 96th hr (as counted from the beginning of culture), when the protein of the present

invention was expressed at highest level on ST2 cells, osteoclast formation was inhibited most effectively. That is, it was revealed that OCIF inhibited osteoclast formation by binding to ST2 cells via the protein of the present invention.

From the above results, it became clear that the protein of the present invention was induced on the membranes of osteoblast-like stromal cells with the active-form of vitamin D₃ and dexamethasone, and had the biological activity (effect) of a factor which supports and promotes differentiation and maturation of osteoclasts.

[Example 6]

Crosslinking Experiment of ¹²⁵I-labeled OCIF to the Protein of the Present Invention

To further identify the presence of the protein of the present invention, ¹²⁵I-labeled OCIF was allowed to crosslink with the protein of the present invention. As in Example 4-(3), mouse osteoblast like cell line, ST2, was cultured in the presence or absence of the active-form of vitamin D₃ and dexamethasone for 4 days. After the cells were washed with 1 ml of phosphate buffered saline solution, 200 µl of medium for binding experiment (α-MEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.2% NaN₃, and 100 µg/ml heparin) further containing 25 ng/ml of the above ¹²⁵I-labeled OCIF (monomer type) or 40 ng/ml of ¹²⁵I-labeled OCIF-CDD1 was added. ¹²⁵I-labeled OCIF-CDD1 was obtained by expressing the protein described as SEQ ID NO: 76 in WO 96/26217 with animal cells and labeling in accordance with the above method. Furthermore, the medium for the binding experiment, containing a 400-fold higher concentration of OCIF, was added to the other well and was subjected to an experiment for nonspecific binding. After the cells were cultured in a CO₂ incubator for 1 hour, they were washed three times with 1 ml of phosphate buffered saline solution containing 100 µg/ml of heparin. Then, 500 µl of phosphate buffered saline solution in which 100 µg/ml of crosslinking agent DSS (Disuccinimidyl suberate, Pierce Co., Ltd.) was dissolved was added thereto, and allowed to react at 0°C for 10 minutes. After the cells in these wells were washed twice with 1 ml of phosphate buffered saline solution cooled to 0°C, 100 µl of 20 mM Hepes buffer containing 1% Triton X-100, 2 mM PMSF (phenylmethylsulfonyl fluoride), 10 µM pepstatin, 10 µM leupeptin, 10 µM antipain and 2 mM EDTA was added to each well, and left to stand at room temperature for 30 minute so as to lyse the cells. After 15 µl of these samples were treated with SDS under non-reducing conditions in accordance with a commonly used method, they were run on a SDS-polyacrylamide electrophoresis gel (with a gradient of 4 to 20% polyacrylamide, Daiichi Pure Chemicals Co., Ltd.). After electrophoresis, the gel was dried and exposed to BIOMAX® MS film (Kodak Co., Ltd.) using BIOMAX® MS amplifying screen (Kodak Co., Ltd.) at -80°C for 24 hours. The exposed films were developed in accordance with a

commonly used method. When the ^{125}I -labeled OCIF (monomer type, 60 kDa) was used, a crosslinked protein having a molecular weight of about 90,000 to 110,000 was detected. On the other hand, when the ^{125}I -labeled OCIF-CDD1 (31 kDa) was used, a crosslinked protein of about 70 to 80 kDa (78 kDa on average) was detected as shown in Fig. 7.

5 [Example 7]

Scatchard Plot Analysis of the Protein of the Present Invention Expressed on ST2 Cells

Medium for the binding experiment (α -MEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer and 0.2% NaN_3) further containing 1,000 pM of the above ^{125}I -labeled OCIF (monomer type) was prepared and diluted stepwise at a dilution rate of 1/2 with the medium for the binding experiment. Furthermore, another medium for determining nonspecific binding was prepared by adding a 400-fold higher concentration of unlabeled monomer type OCIF to the above medium. 200 μl of these prepared solutions were added to wells of the above ST2 cells (about 10^{th} passage), cultured for 4 days in the presence of 10^{-8} M of the active-form of vitamin D_3 (calcitriol) and 10^{-7} M dexamethasone, and binding of the ^{125}I -labeled OCIF was tested in the same manner as in Example 4-(3). The obtained results were Scatchard-plotted in accordance with a common method, and dissociation constants of OCIF and the OCIF binding protein, and the number of the OCIF binding protein (site) per one ST2 cell were determined. As a result, the dissociation constants of OCIF and the protein of the present invention were 280 pM, and the number of the OCIF binding protein (site) per one ST2 cell was about 33,000/cell. Furthermore, a cultured ST2 cell with a passage number of around 40's had higher ability to support osteoclast formation than that with a passage number of around 10's as shown in Example 5-(1), so that the number of sites of the protein of the present invention expressed on the ST2 cell with a passage number of around 40's was measured. As a result, the number of site was 58,000/cell which, was clearly greater than that on the ST2 cell with a passage number of around 10's. It was revealed that the amount of the expression of the protein of the present invention associated with the degree of the ability of ST2 cell to support osteoclast formation. This finding indicates that the protein of the present invention is a factor to support and promote differentiation and maturation of osteoclasts.

30 [Example 8]

Cloning of OBM cDNA

(1) Extraction of RNA from Mouse ST2 Cell

Mouse osteoblast-like stromal cell line, ST2, (Riken Cell Bank, RCB0224) was with α -MEM medium (Gibco BRL Co., Ltd.) containing 10% bovine fetal serum. After cultured cells become confluent in 225- cm^2 T flasks for adherent cell culture, ST2 cells were treated with trypsin, stripped from the T flask, washed, and transferred to five 225- cm^2 T

flasks. After adding 60 ml of α -MEM medium containing 10^{-8} M of the active-form of vitamin D₃ (calcitriol, Wako Pure Chemical Industries, Ltd.), 10^{-7} M dexamethasone and 10% bovine fetal serum thereto, the cells were cultured in a CO₂ incubator for 5 days. Total RNA was extracted from the cultured ST2 cells using ISOGEN (Wako Pure Chemical Industries, Ltd.). Poly A⁺ RNA was prepared from about 600 μ g of the total RNA using an Oligo(dT)-cellulose column (5'-3' Prime Co., Ltd.). About 8 μ g of poly A⁺ RNA was obtained.

(2) Construction of Expression Library

Double strand cDNAs were synthesized from 2 μ g of the poly A⁺ RNA obtained in Example 8-(1) with Great Lengths cDNA Synthesis kit (Clontech Co., Ltd.) in accordance with a manual thereof. More specifically, 2 μ g of the poly A⁺ RNA and an Oligo(dT)₂₅(dN) primer were mixed together, distilled water was added thereto so that the final volume was 6.25 μ l, and the mixture was incubated at 70°C for 3 minutes, and then cooled in ice for 2 minutes. Then, 2.2 μ l of distilled water, 2.5 μ l of 5X First-strand buffer, 0.25 μ l of 100 mM DTT (dithiothreitol), 0.5 μ l of PRIME RNase Inhibitor (1 U/ml) (5'-3' Prime Co., Ltd.), 0.5 μ l of [α -³²P]dCTP (Amersham Co., Ltd., 3,000 Ci/mmol, 2 μ Ci/ μ l) which was diluted to be one fifth concentration, 0.65 μ l of dNTP (20 mM each) and 1.25 μ l (250 units) of MMLV (RNaseH⁻) reverse transcriptase were added thereto, respectively. Thus obtained solution was incubated at 42°C for 90 minutes. Then, 62.25 μ l of distilled water, 20 μ l of 5X second-strand buffer, 0.75 μ l of dNTP (20 mM each) and 5 μ l of Second-strand enzyme cocktail were added thereto, respectively. Thus obtained solution was incubated at 16°C for 2 hours. 7.5 units of T4 DNA polymerase was added thereto, and further incubated at 16°C for another 30 minutes. Thereafter, 5 μ l of 0.2 M EDTA was added to terminate the reaction, and after a phenol-chloroform treatment, ethanol precipitation was carried out. An EcoRI-SalI-NotI linker (Clontech Co., Ltd.) was added to an end of the double strand cDNA and then phosphorylated at its end. Using a column for size fractionation, cDNAs of not smaller than 500 bp were separated, and ethanol-precipitated. The precipitated DNAs were reconstituted in water and inserted into pcDL-SR α 296 (Molecular and Cellular Biology, Vol. 8, pp. 466 to 472, 1988) (Takara Shuzo Co., Ltd.) previously cleaved with a restriction enzyme, EcoRI, and subsequently treated with CIAP (calf intestine alkaline phosphatase, Takara Shuzo Co., Ltd.).

(3) Screening of Expression Library in Which the Binding to OCIF Was Used as an Index

E. coli XL2 Blue MRF' (Toyobo Co., Ltd.) was transformed with the DNA obtained in Example 8-(2), and allowed to grow on a L Carbenicillin Agar Medium (1% trypton, 0.5% yeast extract, 1% NaCl, 60 μ g/ml carbenicillin and 1.5% agar) prepared in a 24-well plastic plate for cell culture so that the cells was grown to about 100 colonies per

well. The transformants in each well were suspended in 3 ml of Terrific Broth ampicillin medium (1.2% trypton, 2.4% yeast extract, 0.4% glycerol, 0.017 M KH_2PO_4 , 0.072 M K_2HPO_4 , 100 $\mu\text{g/ml}$ ampicillin), and cultured with shaking at 37°C overnight. The *E. coli* was collected by centrifugation, and plasmid DNAs were prepared therefrom with

5 QIAWELL® kit (QIAGEN Co., Ltd.). The DNA content was determined by detecting absorbance at 260 nm, and the DNAs was concentrated by ethanol precipitation and dissolved in distilled water so that the concentration was 200 ng/ μl . Thus, 500 DNA pools each derived from about 100 colonies were prepared and used for transfection of COS-7 cells (Riken Cell Bank, RCB0539). COS-7 cells were seeded in a 24-well plate so as to achieve 8

10 $\times 10^4$ cells/well and cultured in a CO_2 incubator at 37°C overnight by use of DMEM medium containing 10% bovine fetal serum. On the following day, the medium was removed, and the cells were then washed with serum-free DMEM medium. In accordance with a protocol attached to lipofectamine (Gibco Co., Ltd.) which was a reagent for transfection, the plasmid DNA previously diluted with OPTI-MEM® medium (Gibco BRL Co., Ltd.) and

15 lipofectamine were mixed together, and after 15-minute incubation, the mixture was added to the cells in each well. The amounts of DNA and lipofectamine used were 1 μg and 4 μl , respectively. After 5-hour incubation, the medium was removed, and 1 ml of DMEM medium (Gibco BRL Co., Ltd.) containing 10% bovine fetal serum was added and cultured in a CO_2 incubator (5% CO_2) at 37°C for 2 to 3 days. The COS-7 cells obtained after

20 transfection and subsequent culture for 2 to 3 days were washed with serum-free DMEM medium. Then, 200 μl of medium for binding experiment (serum-free DMEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.1 mg/ml heparin and 0.02% NaN_3) further containing 20 ng/ml of ^{125}I -labeled OCIF was added thereto. Cells were cultured in a CO_2 incubator (5% CO_2) at 37°C for 1 hour and washed twice with 500 μl of

25 phosphate buffered saline solution containing 0.1 mg/ml heparin. After washing, 500 μl of 0.1 N NaOH solution was added thereto, and then left to stand at room temperature for 10 minutes so as to lise the cells. The amount of ^{125}I in each well was measured with a gamma counter (Packard Co., Ltd.). After screening the 500 pools in total, a DNA pool containing a cDNA encoding a protein that could specifically bind OCIF was isolated. Furthermore, the

30 DNA pools containing the cDNA of the present invention were subfractionated, and then employed to repeat the above transfection and screening. Thereafter, a cDNA encoding a protein which could bind OCIF was isolated. A plasmid containing the cDNA was referenced pOBM291. *E. coli* containing the plasmid was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology as

35 pOBM291 with the deposition number of FERM BP-5953 on May 23, 1997. Methods for

¹²⁵I-labeling of OCIF and determining (the concentration of) ¹²⁵I-labeled OCIF by ELISA are as follows. OCIF was ¹²⁵I-labeled in accordance with Iodogen method. 20 µl of 2.5 mg/ml Iodogen-chloroform solution was transferred to a 1.5 ml Eppendorf tube, and chloroform was evaporated at 40°C so as to prepare an Iodogen-coated tube. After the tube was washed three times with 400 µl of 0.5 M sodium phosphate buffer (Na-Pi, pH 7.0), 0.5 µl of 0.5 M Na-Pi with a pH of 7.0 was added thereto. Immediately after 1.3 µl (18.5 MBq) of Na-¹²⁵I solution (Amersham Co., Ltd., NEZ-033H20) was added thereto, 10 µl of 1 mg/ml OCIF solution (monomer type or dimer type) was added. The resulting solution was agitated with a vortex mixer and then left to stand at room temperature for 30 seconds. This solution was transferred to a tube in which 10 mg/ml potassium iodide, 80 µl of 0.5 M Na-Pi solution (pH 7.0) and 5 µl of phosphate buffered saline solution containing 5% bovine serum albumin (BSA-PBS) was previously added and then agitated. This solution was applied to a spin column (1 ml, G-25 fine, Pharmacia Co., Ltd.) equilibrated with BSA-PBS and centrifuged at 2,000 rpm for 5 minutes. After 400 µl of BSA-PBS was added to an eluate from the column and mixed, 2 µl was subfractionated and its radioactivity was measured with a gamma counter. The radiochemical purity of thus prepared ¹²⁵I-labeled OCIF solution was determined by measuring the radioactivity of a fraction precipitated with 10% TCA. Furthermore, the biological activity as OCIF of the ¹²⁵I-labeled OCIF solution was determined in accordance with a method described in WO 96/26217. Moreover, the concentration of ¹²⁵I-labeled OCIF was measured by ELISA in the following manner. That is, 100 µl of 50 mM NaHCO₃ (pH 9.6) in which 2 µg/ml of anti-OCIF rabbit polyclonal antibody described in WO 96/26217 was dissolved was added to each well of a 96-well immunoplate (Nunc Co., Ltd., MaxiSorp) and left to stand at 4°C overnight. After this solution was removed, 200 µl of a combined solution of of BLOCKACE (Snow Brand Milk Products Co., Ltd.) and phosphate buffered saline solution (mixing ratio = 25:75: B-BPB) was added to each well and then left to stand at room temperature for 2 hours. After this solution was removed, each well was washed three times with phosphate buffered saline solution (P-PBS) containing 0.01% Polysorbate 80. Thereafter, 100 µl of B-PBS containing a ¹²⁵I-labeled OCIF or standard OCIF was added thereto and left to stand at room temperature for 2 hours. After this solution was removed, each well was washed six times with 200 µl of P-PBS. Then, a peroxidase-labeled anti-OCIF rabbit polyclonal antibody was diluted with B-PBS and 100 µl of the diluted solution was added to each well, and then left to stand at room temperature for 2 hours. After this solution was removed, each well was washed six times with 200 µl of P-PBS. Then, 100 µl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well and then left to stand at room

temperature for 2 to 3 minutes. Thereafter, 100 μ l of Stopping Reagent (Scytek Co., Ltd.) was added thereto. The absorbance of each well at 450 nm was measured with a microplate reader. The concentration of the 125 I-labeled OCIF was determined from a calibration curve made using standard OCIF.

5 **(4) Determination of the Nucleotide Sequence of cDNA Which Eccodes the Full Length Amino Acid Sequence of OBM**

 The nucleotide sequence of OBM cDNA obtained in Example 8-(3) was determined with Taq Dye Deoxy Terminator Cycle Sequencing kit (Perkin Elmer Co., Ltd.). That is, using pOBM291 as a template, the nucleotide sequence of the inserted fragment was
10 directly determined. Furthermore, about 1.0 kb and about 0.7 kb fragments obtained by cleaving pOBM291 with a restriction enzyme, EcoRI, were inserted into EcoRI site of plasmid pUC19 (Takara Shuzo Co., Ltd.) and sequenced, respectively. A primer SRR2 for sequencing the DNA fragment inserted in pcDL-SR α 296, primers M13PrimerM3 and M13PrimerRV (Takara Shuzo Co., Ltd.) for sequencing the DNA fragment inserted in the
15 plasmid pUC19, and a synthetic primer OBM #8 designed based on the nucleotide sequence of OBM cDNA were used. The sequences of these primers are shown as SEQ ID NOs: 3 to 6.

 Furthermore, the determined nucleotide sequence of OBM cDNA is shown as SEQ ID NO: 2, and the deduced an amino acid sequence is shown as SEQ ID NO: 1.

[Example 9]

20 **Expression of the Protein Encoded by the cDNA of the Present Invention**

 COS-7 cells were transfected with plasmid pOBM291 with lipofectamine in each well of a 6-well plate, and were cultured in DMEM medium containing 10% bovine fetal serum for 2 days. The medium was replaced with cysteine/methionine-free DMEM (DAINIPPON PHARMACEUTICAL CO., LTD.) in which 5% dialyzed bovine fetal serum
25 (800 μ l/well) was added, and the cells were cultured for another 15 minutes. Then, 14 μ l of Express Protein Labeling Mix (NEN CO., LTD., 10 mCi/ml) was added thereto. After the cells were cultured for 4 hours, 200 μ l of DMEM medium containing 10% bovine fetal serum was added, and the cells were cultured for 1 hour. After the cells were washed twice with
30 PBS, 0.5 ml of TSA buffer (10 mM Tris-HCl (pH 8.0) containing 0.14 M NaCl and 0.025% NaN₃) containing 1% Triton X-100, 1% bovine hemoglobin, 10 μ g/ml leupeptin, 0.2 TIU/ml aprotinin and 1 mM PMSF was added, and the cells were left to stand on ice for 1 hour. After the cells were crushed by pipetting, centrifugation was carried out at 3,000 xg at 4°C and for 10 minutes so as to obtain a supernatant. To 100 μ l of this supernatant, 200 μ l of
35 dilution buffer (TSA buffer containing 0.1% Triton X-100, 0.1% bovine hemoglobin, 10 μ g/ml leupeptin, 0.2 TIU/ml aprotinin and 1 mM PMSF) was added, and the resulting

supernatant was shaken together with Protein A Sepharose® (50 µl) at 4°C for 1 hour, and then centrifuged at 4°C and 1,500 X g for 1 minute so as to collect a supernatant. Thereby, a fraction nonspecifically binding to the Protein A Sepharose® was removed. OCIF (1 µg) was added to this supernatant, and the obtained supernatant was shaken at 4°C for 1 hour so that OBM bound OCIF. Then, an anti-OCIF polyclonal antibody (50 µg) was added, and the solution was shaken at 4°C for 1 hour. Then, Protein A Sepharose® (10 µl) was further added, and the solution was further shaken at 4°C for another 1 hour. The solution was centrifuged at 1,500 xg at 4°C for 1 minute and the precipitated fraction was collected. The precipitate resulting from centrifugation at 1,500 xg at 4°C for 1 was washed twice with the dilution buffer, twice with the dilution buffer without bovine hemoglobin, once with TSA buffer, and once with 50 mM Tris-HCl (pH 6.5). After washing, SDS buffer (0.125 M Tris-HCl, 4% dodecyl sodium sulfate, 20% glycerol, 0.002% bromophenol blue, pH 6.8) containing 10% β mercaptoethanol was added to the precipitate. The precipitate was heated at 100°C for 5 minutes and subjected to SDS-PAGE (12.5% polyacrylamide gel, Daiichi Pure Chemicals Co., Ltd.). After the gel was fixed in accordance with a commonly used method, signals of isotope were amplified with Amplify (Amersham Co., Ltd.), and the fixed gel was exposed to BioMax® MR film (Kodak Co., Ltd.) at -80°C. The results are shown in Fig. 8. The molecular weight of the protein encoded by the cDNA of the present invention was found to be about 40,000.

[Example 10]

Binding of the Protein Encoded by the cDNA of the Present Invention to OCIF

COS-7 cells were transfected with plasmid pOBM291 with lipofectamine in wells of a 24-well plate and cultured for 2 to 3 days. Then, the cells were washed with serum-free DMEM medium, and 200 µl of medium for binding experiment (serum-free DMEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.1 mg/ml heparin and 0.2% NaN₃) containing 20 ng/ml of ¹²⁵I-labeled OCIF was added thereto. Furthermore, 200 µl of the medium for binding experiment containing 8 µg/ml of unlabeled OCIF in addition to 20 ng/ml of the ¹²⁵I-labeled OCIF was added to other wells. The cells were cultured in a CO₂ incubator (5% CO₂) at 37°C for 1 hour, and washed twice with 500 µl of phosphate buffered saline solution containing 0.1 mg/ml heparin. After washing, 500 µl of 0.1 N NaOH solution was added to each well, and then the well was left to stand at room temperature for 10 minutes so as to lyse the cell. The amount of ¹²⁵I in each well was measured with a gamma counter. As a result, it was confirmed that the ¹²⁵I-labeled OCIF bound only to cells transfected with the plasmid pOBM291 as shown in Fig. 9. Further, it was also confirmed that the binding was significantly inhibited by addition of a 400-fold

higher concentration of (unlabeled) OCIF. From these results, it was revealed that OBM, a protein encoded by the cDNA of the plasmid pOBM291 specifically bound OCIF on the surface of COS-7 cell.

[Example 11]

5 **Crosslinking Experiment of ^{125}I -labeled OCIF to the Protein Encoded by the cDNA of the Present Invention**

In order to analyze the characteristics of the protein encoded by the cDNA of the present invention more specifically, ^{125}I -labeled monomer type OCIF was allowed to crosslink with the protein encoded by the cDNA of the present invention. COS-7 cells were
10 transfected with plasmid pOBM291 in accordance with the method described in Example 8-(3), 200 μl of medium for binding experiment containing the above ^{125}I -labeled OCIF (25 ng/ml) was added thereto. Furthermore, the medium for the binding experiment containing a 400-fold higher concentration of unlabeled OCIF in addition to the ^{125}I -labeled OCIF was added to other wells. The cells were cultured in a CO_2 incubator (5% CO_2) at 37°C for 1
15 hour and washed twice with 500 μl of phosphate buffered saline solution containing 0.1 mg/ml of heparin. 500 μl of phosphate buffered saline solution containing 100 $\mu\text{g/ml}$ of crosslinking agent DSS (Disuccinimidyl suberate, Pierce Co., Ltd.) was added to these cells, and the cells were allowed to react at 0°C for 10 minutes. After the reaction, the cells in these wells were washed twice with 1 ml of phosphate buffered saline solution cooled to 0°C .
20 Then, 100 μl of 20 mM Hepes buffer containing 1% Triton X-100 (Wako Pure Chemical Industries, Ltd.), 2 mM PMSF (phenylmethylsulfonyl fluoride, Sigma Co., Ltd.), 10 μM pepstatin (Wako Pure Chemical Industries, Ltd.), 10 μM leupeptin (Wako Pure Chemical Industries, Ltd.), 10 μM antipain (Wako Pure Chemical Industries, Ltd.) and 2 mM EDTA (Wako Pure Chemical Industries, Ltd.) was added to these cells, and the wells were left to
25 stand at room temperature for 30 minute so as to lise the cells. After 15 μl of these samples were treated with SDS under non-reducing conditions in accordance with a commonly used method, they were subjected to electrophoresis with gel for SDS-electrophoresis (gradient of 4 to 20% polyacrylamide, DAIICHI PURE CHEMICALS CO., LTD.). After electrophoresis, the gel was dried and exposed to BioMax® MS film (Kodak Co., Ltd.) with BioMax® MS
30 amplifying screen (Kodak Co., Ltd.) at -80°C for 24 hours. The exposed films were developed in accordance with a commonly used method. As results of crosslinking of the ^{125}I -labeled monomer type OCIF with the protein encoded by the cDNA of the present invention, a band having a molecular weight of about 90,000 to 110,000 was detected as shown in Fig. 10.

35 [Example 12]

Northern Blot Analysis

ST2 cells were cultured to become confluent in a 25-cm² T flask, for culturing adherent cells, and treated with trypsin. After being stripped from the T flask, the cells were washed and seeded in a 225-cm² T flask. 60 ml of α -MEM medium containing 10⁻⁸ M of the active-form of vitamin D₃ 10⁻⁷ M dexamethasone and 10% bovine fetal serum was added thereto, and the cells were cultured in a CO₂ incubator for 4 days. Then, total RNA was extracted from the above-cultured ST2 cells with ISOGEN (Wako Pure Chemical Industries, Ltd.). In addition, total RNA was extracted from ST2 cells cultured in the absence of the active-form of vitamin D₃ and dexamethasone in accordance with the above method. To 20 μ g (4.5 μ l) of each total RNA sample, 2.0 μ l of 5X gel electrophoresis buffer (0.2 M morpholinopropanesulfonic acid (pH 7.0), 50 mM sodium acetate, 5 mM EDTA), 3.5 μ l of formaldehyde and 10.0 μ l of formamide were added. The total RNA samples were incubated at 55°C for 15 minutes and subjected to electrophoresis. Gels for electrophoresis consisted of 1.0% agarose, 2.2 M ionized formaldehyde, 40 mM morpholinopropanesulfonic acid (pH 7.0), 10 mM sodium acetate and 1 mM EDTA. Moreover, the electrophoresis was performed in buffer comprising 40 mM morpholinopropanesulfonic acid (pH 7.0), 10 mM sodium acetate and 1 mM EDTA. After the electrophoresis, the RNA was transferred to nylon membranes. About 1.0 kb DFA fragments were obtained by cleaving pOBM291 with a restriction enzyme, EcoRI, and labeled with α -³²P-dCTP (Amersham Co., Ltd.) using MEGAPRIME DNA Labeling Kit (Amersham Co., Ltd.), and thus used as probes for hybridization. As a result, it was revealed that gene expression of the protein (OBM) encoded by the cDNA of the present invention was strongly induced in the ST2 cells cultured in the presence of the active-form of vitamin D₃ and dexamethasone.

[Example 13]

Ability of the Protein Encoded by the cDNA of the Present Invention to Support Osteoclast Formation

In accordance with the method described in Example 8-(3), COS-7 cells were transfected with pOBM219. After 3-day incubation, the cells were treated with trypsin and then centrifuged-washed once with phosphate buffered saline solution. Then, the cells were fixed at room temperature for 5 minutes in suspension of PBS containing 1% paraformaldehyde, and then centrifuged-washed six times with PBS. Mouse spleen cells and ST2 cells were prepared with α -MEM medium containing 10⁻⁸M of the active-form of vitamin D₃, 10⁻⁷M dexamethasone and 10% bovine fetal serum so that the cell concentration become 1 X 10⁶ cells/ml or 4 X 10⁴ cells/ml and then added to a 24-well plate in a volume of 700 μ l and 350 μ l, respectively. Furthermore, TC insert (Nunc Co., Ltd.) was set in each

well. The fixed COS cells (350 µl) diluted stepwise with the above medium and OCIF (50 µl), were added to TC inserts and cultured at 37°C for 6 days. As a result, it was revealed that an activity of OCIF to inhibit osteoclast formation was suppressed by the protein encoded by the cDNA of the present invention.

[Example 14]

Expression of Secretory-type OBM

(1) Construction of Plasmid for Expressing Secretory-Type OBM Expression

A PCR reaction was carried out using OBM HF (SEQ ID NO: 7)/OBM XR (SEQ ID NO: 8) and pOBM291 as primers and a template, respectively. After the reaction product was purified through agarose gel electrophoresis, it was digested with the restriction enzymes, HindIII and EcoRI, and then purified through agarose gel electrophoresis again. The purified fragment (0.6 kb), HindIII/EcoRV fragment (5.2 kb) of pSec TagA (Invitrogen Co., Ltd.) and EcoRI/PmaCI fragment (0.32 kb) of OBM cDNA was subjected to ligation using Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.), and subsequently *E. coli* DH5α was transformed by the ligation product. Plasmid was purified from the obtained ampicillin-resistant strains by alkaline-SDS method and then cleaved with restriction enzymes so as to select a plasmid wherein 0.6 kb and 0.32 kb of fragments were inserted into pSec TagA. The selected plasmid was subjected to sequencing with Dye Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.), thereby it was confirmed that the plasmid had the sequence encoding secretory-type OBM (nucleotides 338-1355 of SEQ ID NO: 2, amino acids 72-316 of SEQ ID NO: 1). After the plasmid was digested with restriction enzymes, NheI and XhoI, a fragment (1.0 kb) corresponding to secretory-type OBM cDNA was collected by agarose gel electrophoresis. This fragment was inserted into a NheI/XhoI digested expression vector, pCEP4 (10.4 kb) (Invitrogen Co., Ltd.), by using the ligation kit, and *E. coli* DH5α were transformed with the ligation product. Plasmid was purified, from the ampicillin-resistant strains obtained, by alkaline-SDS method, and digested with the restriction enzymes. Then the plasmid was analyzed so as to select *E. coli* strains which had a plasmid for expressing secretory-type OBM (pCEP sOBM) with the desired structure. An *E. coli* strain having the pCEP sOBM was cultured, and the pCEP sOBM was purified therefrom with QIA® Filter Plasmid Midi Kit (QIAGEN CO., LTD.).

(2) Expression of Secretory-Type OBM

293-EBNA cells were suspended in IMDM containing 10% FCS (IMDM-10% FCS), and seeded in a collagen-coated 24 well plate (SUMITOMO BAKELITE CO., LTD.) so that the cell concentration was 2×10^5 cells/2 ml/well, and cultured overnight. The cells were transfected with 1 µg of pCEP sOBM or pCEP4 using 4 µl of lipofectamine

(GIBCO CO., LTD.), and then cultured in 0.5 ml of serum-free IMDM or IMDM-10% FCS for another 2 days. Thereafter, the conditioned medium was collected. Expression of secretory-type OBM in the conditioned medium was confirmed in the following manner. After sodium hydrogencarbonate was added to the conditioned medium so that the final concentration was 0.1 M, the culture solution was added to a 96-well plate, and left to stand at 4°C overnight. Then the OBM in the conditioned medium was immobilized in the 96-well plate. This plate was left to stand for blocking at room temperature for 2 hours by use of BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution diluted with PBS to be one forth concentration (B-PBS). Then, 100 µl of 3-100 ng/ml OCIF diluted with B-PBS was added to each well, and the wells were left to stand at 37°C for 2 hours. After washing the plate with PBS containing 0.05% Tween 20 (PBS-T), 100 µl of peroxidase-labeled anti-OCIF rabbit polyclonal antibody, which was described in WO 96/26217, diluted with B-PBS was added to each well, and the cells were left to stand at 37°C for 2 hours. After washing each well with PBS-T six times, 100 µl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added thereto and then left to stand at room temperature for about 10 minutes. Thereafter, 100 µl of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at 450 nm was measured with a microplate reader. The results are shown in Fig. 12. In the plate in which substances included in the conditioned medium of the cells transduced by pCEP sOBM were immobilized, absorption at 450 nm increased in the OCIF concentration-dependent manner. On the other hand, in the plate in which substances included in the conditioned medium of the cells transduced by pCEP4 vector were immobilized, no increase in absorption at 450 nm was observed. Furthermore, Fig. 13 shows the results of experiments when the amount of the conditioned medium applied to the immobilization was varied within a range of 5 to 90% and a constant concentration of OCIF (50 ng/ml) was further added. In the plate in which substances included in the conditioned medium of the cells transduced by pCEP sOBM were immobilized, absorption at 450 nm increased corresponding to an increase in the amount of the conditioned medium. On the other hand, in the plate in which substances included in the conditioned medium of the cells transduced by pCEP4 vector were immobilized, no increase in absorption was observed. From these results, secretory-type OBM was confirmed to be produced in the conditioned medium of the cells transduced by pCEP sOBM.

[Example 15]

Expression of Thioredoxin-OBM Fusion Protein (Trx-OBM)

(1) Construction of a Vector for Expressing Thioredoxin-OBM Fusion Protein (Trx-OBM)

10 µl of 10X ExTaq buffer (TAKARA SHUZO CO., LTD.), 8 µl of 10 mM

dNTPS (TAKARA SHUZO CO., LTD.), 77.5 µl of sterilized distilled water, 2 µl of pOBM291 solution (10 ng/µl), 1 µl of primer OBM3 (100 pmol/µl, SEQ ID NO: 9), 1 µl of primer OBMSalR2 (100 pmol/µl, SEQ ID NO: 10) and 0.5 µl of ExTaq (5µ/µl) (Takara Shuzo Co., Ltd.) were mixed together, and then PCR was conducted in a microtube for centrifugation. After the reaction was carried out at 95°C for 5 minutes, 50°C for 1 second, 55°C for 1 minute, 74°C for 1 second and 72°C for 5 minutes, the cycle reaction consisting of at 96°C for 1 minute, 50°C for 1 second, 55°C for 1 minute, 74°C for 1 second and 72°C for 3 minutes was repeated 25 times. After gel electrophoresis through 1% agarose, an approximately 750 bp DNA fragment was purified from the whole reaction solution with QIAEX® II Gel Extraction Kit (QIAGEN Co., Ltd.). All of the purified DNA fragment was cleaved with restriction enzymes SalI and EcoRI (Takara Shuzo Co., Ltd.), and a DNA fragment about 160-bp (fragment 1) was purified by gel electrophoresis through 1.5% agarose and dissolved in 20 µl of sterilized distilled water. Similarly, 4 µg of pOBM291 and 2 µg of pTrxFus (Invitrogen Co., Ltd.) were cleaved with restriction enzymes BamHI/EcoRI and BamHI/SalI (Takara Shuzo Co., Ltd.), respectively. A DNA fragment about 580-bp (fragment 2) and an approximately 3.6-kb DNA fragment (fragment 3) were purified therefrom, respectively, and dissolved in 20 µl of sterilized distilled water. QIAEX® II Gel Extraction Kit was used for purifying the fragments. Fragments 1, 2 and 3 were ligated by incubating them using DNA Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.) at 16°C for 2.5 hours. Then, *E. coli* GI724 cells (Invitrogen Co., Ltd.) were transformed with the ligation product in accordance with the method described in an instruction manual attached to ThioFusion Expression System (Invitrogen Co., Ltd.). Among the resulting ampicillin-resistant transformants, one having a plasmid, in which an OBM cDNA fragment (350-1111 of SEQ ID NO: 2, corresponding to: 76-316 of SEQ ID NO: 1) was linked to a thioredoxin gene in the same reading frame and was selected after analysis of DNA fragment map obtained by restriction enzyme cleavage and DNA sequencing. The obtained strain was referenced as GI724/pTrxOBM25.

(2) Expression of OBM in *E. coli*

The GI724/pTrxOBM25 strain and the GI724 strain having pTrxFus (GI724/pTrxFus) were cultured in 2 ml of RMG-Amp medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 1.2% casamino acid (Difco Co., Ltd.), 1% glycerol, 1 mM MgCl₂, 100 µg/ml ampicillin (Sigma Co., Ltd.), pH 7.4) with shaking at 30°C for 6 hours. 0.5 ml of the cell suspension was added to 50 ml of Induction medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 0.2% casamino acid, 0.5% glucose, 1 mM MgCl₂, 100 µg/ml ampicillin, pH 7.4) and cultured with shaking at 30°C. L-

tryptophan was added so that the final concentration was 0.1 mg/ml when the value at OD₆₀₀ became about 0.5, and the cells were further cultured at 30°C for 6 hours. The cell suspension was centrifuged at 3,000 X g and the collected cells were then suspended in 12.5 ml of PBS (10 mM phosphoric acid buffer, 0.15 M NaCl, pH 7.4). The suspension was subjected to ultrasonication using an ultrasonicator (Ultrasonics Co., Ltd.) so that the cells were crushed and then centrifuged at 7,000 X g for 30 minutes. The recovered supernatant was used as soluble protein fraction. 10 µl of the soluble protein fraction solution was subjected to SDS polyacrylamide (10%) electrophoresis under reducing conditions. As a result, a band having a molecular weight of about 40 kDa was observed in the soluble protein fraction solution of GI724/pTrxOBM25, while not observed in soluble protein fraction solution of GI724/pTrxFus (Fig. 14). Thus, it was confirmed that the thioredoxin-OBM fusion protein (Trx-OBM) was expressed in *E. coli*.

(3) Binding Ability of Trx-OBM to OCIF

In the following experiment, it was confirmed that the expressed Trx-OBM bound to OCIF. Anti-thioredoxin antibody (Invitrogen Co., Ltd.) was diluted with 10 mM sodium hydrogencarbonate solution so that the concentration was 1/5,000. 100 µl thereof was added to each well of a 96-well immunoplate (Nunc Co., Ltd.) and then left to stand at 4°C overnight. After the solution in each cell was discarded, 200 µl of 1/2 concentration of BLOCKACE (Snow Brand Milk Products Co., Ltd.) diluted with PBS (BA-PBS) was added to each well and then left to stand at room temperature for 1 hour. After the solution was discarded, 100 µl of the soluble protein fraction solution derived from GI724/pTrxOBM25 which was diluted stepwise with BA-PBS and 100 µl of that derived from GI724/pTrxFus which was diluted stepwise with BA-BPB were added to wells and left to stand at room temperature for 2 hours, respectively. After washing each well three times with PBS-T, 100 µl of OCIF (100 ng/ml) diluted with BA-PBS was added to each well and left to stand at room temperature for 2 hours. After washing each well three times with PBS-T, 100 µl of peroxidase-labeled anti-OCIF rabbit polyclonal antibody described in WO 96/26217, which was diluted with BA-PBS so that the concentration was 1/2,000, was added to each well and left to stand at room temperature for 2 hours. After washing each well six times with PBS-T, 100 µl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added thereto and then left to stand at room temperature for about 10 minutes. Thereafter, 100 µl of Stopping Reagent (Scytek Co., Ltd.) was added thereto. Absorbance of each well at 450 nm was measured with a microplate reader. The results are shown in Fig. 15. When the concentration of the soluble protein fraction solution derived from GI724/pTrxFus increased, the absorbance increased in a concentration (of the added solution)-dependent

manner, while no difference in absorbance was observed between when the soluble protein fraction solution derived from GI724/pTrxFus was added and when said soluble protein fraction solution was not added. Furthermore, Fig. 16 shows the results of experiments when the dilution rate of the soluble fraction solution was kept constant (1%) and OCIF diluted stepwise with BA-PBS (0-100 ng/ml) was further added. Absorbance was kept low regardless of the concentration of OCIF when soluble protein fraction solution derived from GI724/pTrxFus was added. However, absorbance was increased in an OCIF concentration-dependent manner when soluble protein fraction solution derived from GI724/pTrxOBM25 was added. Thus, it was confirmed that the Trx-OBM produced in GI724/pTrxOBM25 had an ability to bind OCIF.

(4) Large Scale Culture of *E. coli* Producing Trx-OBM

GI724/pTrxOBM25 was spread on an RMG-Amp agar medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 2% casamino acid, 1% glycerol, 1 mM MgCl₂, 100 µg/ml ampicillin, 1.5% agar, pH 7.4) with a platinum loop and cultured at 30°C overnight. The cells were suspended in 10 ml of Induction medium. 5 ml of the suspension was added to two of 2L conical flasks containing 500 ml of Induction medium and cultured by shaking at 30°C. L-tryptophan was added so that the final concentration was 0.1 mg/ml when OD₆₀₀ value became about 0.5, and then the cells were further cultured by shaking at 30°C for 6 hours. The cell suspension was centrifuged at 3,000 X g for 20 minutes, and the cells were collected and then suspended in 160 ml of PBS. The suspension was subjected to ultrasonication using an ultrasonicator (Ultrasonics Co., Ltd.) for crushing cells, and then centrifuged at 7,000 X g for 30 minutes. Thereafter, the supernatant was recovered as soluble protein fraction.

(5) Preparation of OCIF-immobilized Affinity Column

2 g of TSKgel AF-Tresyl TOYOPAL 650 (Toso Co., Ltd.) and 40 ml of 1.0 M potassium phosphate buffer (pH 7.5) containing 35.0 mg of recombinant OCIF prepared by a method described in WO 96/26217 were mixed together and gently shaken at 4°C overnight so as to cause a coupling reaction. After the supernatant was removed by centrifugation, 40 ml of 0.1 M Tris-hydrochloric acid buffer (pH 7.5) was added to the precipitated carrier, and the mixture was gently shaken at room temperature for 1 hour, in order to inactivate an excess amount of active groups thereon. After washing the column with both 0.1 M glycine-hydrochloric acid buffer (pH 3.3) containing 0.01% Polysorbate 80/0.2 M NaCl and 0.1 M sodium citrate buffer (pH 2.0) containing 0.01% Polysorbate 80/0.2 M NaCl, the column was washed twice with 10 mM sodium phosphate buffer (pH 7.4) containing 0.01% Polysorbate 80 and equilibrated therewith.

(6) Purification of Trx-OBM Using OCIF-Immobilized Affinity Column

Purification of Trx-OBM was carried out at 4°C unless otherwise stated. The above OCIF-immobilized affinity carrier (10 ml) and the above soluble protein fraction solution (120 ml) described in Example 15-(4) were mixed together. The mixture was gently shaken in four 50 ml centrifugation tubes with a rotor at 4°C overnight. The carrier in the mixture was embedded in Econo-Column (Bio-Rad Co., Ltd., internal diameter: 1.5 cm, length: 15 cm). The column was washed with 300 ml of PBS containing 0.01% Polysorbate 80, 100 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 0.01% Polysorbate 80 and 2 M NaCl and 100 ml of 0.1 M glycine-hydrochloric acid buffer (pH 3.3) containing 0.01% Polysorbate 80 and 0.2 M NaCl. Then, protein was eluted from the column with 0.1 M sodium citrate buffer (pH 2.0) containing 0.01% Polysorbate 80 and 0.2 M NaCl. 5 ml eluate fractions were collected. Immediately, a 10% volume of 2 M Tris solution (pH 8.0) was added for neutralization. The presence or absence of Trx-OBM in each fraction of the eluate was examined in accordance with the above method as described in Example 15-(3) (the ability to bind OCIF). Fractions containing Trx-OBM were collected and further purified.

(7) Purification of Trx-OBM by Gel Filtration

Using Centriplus® 10 and Centricon® 10 (Amicon Co., Ltd.), about 25 ml of the above Trx-OBM fraction of Example 15-(6) was concentrated by centrifugation to a final volume of about 0.5 ml. This sample was subjected to Superose® 12 HR 10/30 column (1.0 X 30 cm, Pharmacia Co., Ltd.) previously equilibrated with PBS containing 0.01% Polysorbate 80. The column was developed using PBS containing 0.01% Polysorbate 80 as a mobile phase at a flow rate of 0.25 ml/min and 0.25 ml eluate fractions were collected from the column. Trx-OBM in the fractions was detected by the method described in Example 15-(3) and by SDS-polyacrylamide electrophoresis (gradient gel of 10 to 15% polyacrylamide, Pharmacia Co., Ltd.) using Phast System (Pharmacia Co., Ltd.) and silver staining. Fractions (Fr. 20 to 23) containing purified Trx-OBM were collected and subjected to measurement of Trx-OBM protein concentration. The measurement was carried out with DC-protein assay kit (Bio-Rad Co., Ltd.) using bovine serum albumin as a standard.

[Example 16]

Osteoclastogenesis Promoting Activity of OBM

COS-7 cells were transfected with pOBM291 and pcDL-SR α 296 using lipofectamine (Gibco BRL Co., Ltd.), respectively. After the cells were cultured in DMEM containing 10% FCS for 1 day, they were treated with trypsin, and seeded in a 24-well plate, in which a cover glass (15 mm round, Matsunami Co., Ltd.) was seated, so that the concentration became 5×10^4 cells/well. The cells were then cultured for another two days. After washing the culture plate once with PBS, PBS containing 1% paraformaldehyde was added thereto, and the cells were incubated at room temperature for 8 minutes and fixed.

After washing the plate in which the cells were fixed six times with PBS, 700 μ l of 1×10^6 cells/ml suspension of mouse spleen cell in α -MEM containing 10^{-8} M of the active-form of vitamin D₃, 10^{-7} M dexamethasone and 10% bovine fetal serum was added to each cell. A Millicell® PCF (Millipore Co., Ltd.) was set on each well, and 700 μ l of 4×10^4 cells/ml suspension of ST2 cells in the above medium was added to the Millicell® PCF and cultured at 37°C for 6 days. After the culture, the Millicell® PCF was removed, and the plate was washed once with PBS. The cells were fixed with acetone-ethanol solution (50:50) for 1 minute, and then only the cells showing tartaric acid resistant acid phosphatase activity (TRAP activity), a specific marker for osteoclasts, were stained with leukocyte acid phosphatase kit (Sigma Co., Ltd.). As a result of observation using a microscope, 45 ± 18 (average \pm standard deviation, $n = 3$) TRAP positive cells were observed in the wells in which pOBM291-transfected COS-7 cells were fixed, while no cells showing TRAP activity were detected in the wells in which pcDL-SR α 296-transfected COS-7 cells were fixed. Furthermore, it was also confirmed that calcitonin bound said TRAP positive cells. Thereby, it was revealed that OBM had an activity to promote osteoclast formation.

[Example 17]

Osteoclastogenesis Promoting Activities of Trx-OBM and Secretory-type OBM

Mouse spleen cells were suspended in α -MEM containing 10^{-8} M of the active-form of vitamin D₃, 10^{-7} M dexamethasone and 10% bovine fetal serum in a concentration of 2×10^6 cells/ml, and 350 μ l of this suspension was added to each well of a 24-well plate. 350 μ l of the solution (40 ng/ml) obtained by diluting the purified Trx-OBM with the above medium, 350 μ l of solution obtained by diluting the conditioned medium of 293-EBNA cells (which were transduced by pCEP sOBM or pCEP4 cultured in IMDM-10% FCS with the above medium) so that the concentration was 1/10, or 350 μ l of the above medium alone was added to each well. Then, a Millicell® PCF (Millipore Co., Ltd.) was set on each well, and 600 μ l of 4×10^4 cells/ml suspension of ST2 cell in the above medium were added to the Millicell® PCF. After the cells were cultured for 6 days, the Millicell® PCF was removed, and the plate was washed once with PBS. When the cells were fixed with acetone-ethanol solution (50:50) for 1 minute, only the cells showing tartaric acid resistant acid phosphatase activity (TRAP activity) were stained with LEUKOCYTE ACID PHOSPHATASE kit (Sigma Co., Ltd.). Through observation under a microscope, 106 ± 21 (average \pm standard deviation, $n = 3$) TRAP positive cells were observed in the wells when Trx-OBM was added, while no cells showing TRAP activity were detected in the wells when not added. Similarly, 120 ± 31 (average \pm standard deviation, $n = 3$) TRAP positive cells were observed in the wells when the conditioned medium of 293-EBNA cells transduced by pCEP-sOBM was

added, while no cells showing TRAP activity were detected in the wells when not added. Furthermore, it was confirmed that calcitonin bound to these TRAP positive cells. Thereby, it was revealed that Trx-OBM and secretory-type OBM had an activity to promote osteoclast formation.

[Example 18]

Identity of the Protein OBM Expressed by the cDNA of the Present Invention and Natural-Type OCIF Binding Protein of the Present Invention

(1) Preparation of Anti-OBM Rabbit Polyclonal Antibody

Three male Japanese white rabbits (weight: 2.5 to 3.0 kg, purchased from Kitayama Labeth Co., Ltd.) were subjected to hypodermic immunization with 1 ml of emulsion prepared by mixing 200 µg/ml of purified OBM (thioredoxin-OBM fusion protein), which was obtained in accordance with the methods described in Examples 14-(6) and 14-(7), with 200 µg/ml of Freund's complete adjuvant (Difco Co., Ltd.). The immunization was carried out 6 times in total with one-week interval each, and all the blood was collected from the rabbits on the 10th day counted from the last immunization. An antibody was purified from the fractionated serum in the following manner. That is, the antiserum diluted with PBS to be 1/2 concentraion, and ammonium sulfate was added thereto so that the final concentration was 40% (w/v). Then, the antiserum was left to stand at 4°C for 1 hour and centrifuged at 8,000 X g for 20 minutes. Thereafter, the precipitate was collected and dissolved in a small aliquot of PBS, and then dialyzed against PBS at 4°C. The resulting solution was charged onto a Protein G-Sepharose® column (Pharmacia Co., Ltd.). After washing the column with PBS, immunoglobulin G adsorbed was eluted with 0.1 M glycine-hydrochloric acid buffer (pH 3.0), and the pH thereof was immediately adjusted to be neutral with 1.5 M Tris-hydrochloric acid buffer (pH 8.7). After the eluted protein fraction was dialyzed against PBS, absorbance at 280 nm was measured and its concentration was determined (E^{1%} 13.5). Horseradish peroxidase-labeled anti-OBM antibody was prepared with Maleimide Activated Peroxidase Kit (Pierce Co., Ltd.). That is, 80 µg of N-succinimide-S-acetylthioacetic acid was added to 1 mg of the purified antibody and allowed to react at room temperature for 30 minutes. 5 mg of hydroxylamine was added thereto for deacetylation, and then the modified antibody was fractionated by using a polyacrylamide desalting column. The protein fraction was mixed with 1 mg of maleimide activated peroxidase and allowed to react at room temperature for 1 hour, and then the enzyme-labeled antibody was obtained.

(2) Inhibition of Specific Binding of the Protein Expressed by the cDNA of the Present Invention (OBM) or Natural-Type Protein of the Present Invention to OCIF by Anti-OBM Rabbit Polyclonal Antibody

2 µg/ml of purified OBM (thioredoxin-OBM fusion protein) obtained in accordance with the methods described in Examples 15-(6) and 15-(7) and 2 µg/ml of natural-type purified OCIF binding protein of Example 2-(4) were dissolved in 0.1 M sodium hydrogencarbonate, respectively. 100 µl of each solution was added to each well of a 96-well immunoplate (Nunc Co., Ltd.) and then left to stand at 4°C overnight. 200 µl of 50% BLOCKACE was added to each well and left to stand at room temperature for 1 hour. After washing wells three times with PBS containing 0.1% Polysorbate 20 (P20-PBS), 200 µg/ml of anti-OBM rabbit antibody was dissolved in 25% BLOCKACE diluted with P20-PBS, and 100 µl of the antibody solution or 25% BLOCKACE without antibody was added to each well and incubated at 37°C for 1 hour. After washing wells three times with P20-PBS, 100 µl of medium for the binding experiment (P20-PBS containing 0.2% bovine serum albumin, 20 mM Hepes and 0.1 mg/ml Heparin) containing 20 ng/ml of the ¹²⁵I-labeled OCIF described in Example 8-(3) was added thereto. Furthermore, 100 µl of medium for the binding experiment containing 8 µg/ml of unlabeled OCIF in addition to 20 ng/ml of the ¹²⁵I-labeled OCIF was added to other wells. After incubating the immunoplate at 37°C for 1 hour, each well was washed six times with P20-PBS. The amount of ¹²⁵I in each well was measured with a gamma counter. The results are shown in Fig. 17. As shown in Fig. 17, neither OBM obtained by expressing the cDNA of the present invention and subsequently purifying or the natural-type protein of the present invention, which specifically binds OCIF, bound the ¹²⁵I-labeled OCIF when treated with the anti-OBM polyclonal rabbit antibody. On the other hand, it was confirmed that both proteins bound to the ¹²⁵I-labeled OCIF when not treated with said antibody. Furthermore, it was also revealed that bindings of both proteins to OCIF were specific binding since the bindings were significantly inhibited by addition of a 400-fold higher concentration of unlabeled OCIF (8 µg/ml). From the above results, it was revealed that the anti-OBM rabbit polyclonal antibody recognized both OBM which was a protein expressed by the cDNA of the present invention and the natural-type OCIF binding protein of the present invention, and inhibited specific binding of both proteins to OCIF.

[Example 19]

Cloning of Human OBM cDNA

(1) Preparation of Mouse OBM Primer

For screening of human OBM cDNA, a mouse OBM primer prepared in accordance with the method of the above Example, OBM #3 and OBM #8 were used. Sequences thereof are shown in SEQ ID NO: 9 and SEQ ID NO: 6.

(2) Acquisition of Human OBM cDNA Fragments by PCR

A human OBM cDNA fragment was obtained by PCR method using Human Lymph Node Marathon ready cDNA (Clontech Co., Ltd.) which was a human lymph node derived cDNA library as a mold and using the mouse OBM cDNA primer prepared in the above (1).

5	The following are the conditions used for PCR.		
	10X EX Taq buffer (Takara Shuzo Co., Ltd.)	2.0 μ l	
	2.5 mM dNTP	1.6 μ l	
	cDNA solution	1.0 μ l	
	EX Taq (Takara Shuzo Co., Ltd.)	0.2 μ l	
10	Distilled Water	14.8 μ l	
	40 μ M Primer OBM #3	0.2 μ l	
	40 μ M Primer OBM #8	0.2 μ l	

After the above solutions were mixed together in a microfuge tube, PCR was
15 conducted under the following conditions. A pretreatment was carried out at 95°C for 2
minutes, then the cycle reaction consisting of 95°C for 30 seconds, 57°C for 30 seconds and
72°C for 2.5 minutes was repeated 40 times, and the solution was incubated at 72°C for an
approx. 5 minutes. A subfraction of the reaction product and run through agarose by
electrophoresis detected an approximate 690 bp DNA fragment amplified with the above
20 mouse OBM cDNA primers.

(3) Purification of Human OBM cDNA Amplified by PCR and Determination of Nucleotide Sequence

The human OBM cDNA fragments obtained in Example 19-(2) were separated
by agarose gel electrophoresis and then purified by use of a QIAEX® gel extraction kit
25 (QIAGEN Co., Ltd.). By use of the purified human OBM cDNA fragments as templates,
PCR was conducted again by use of the above mouse OBM cDNA primer so as to prepare a
large amount of human OBM cDNA fragments which were then purified by use of the
QIAEX® gel extraction kit. The nucleotide sequence of the purified human OBM cDNA
fragment was determined by use of a Taq Dye Deoxy Terminator Cycle Sequencing FS kit
30 (Perkin Elmer Co., Ltd.) using OBM #3 and OBM #8 (SEQ ID NO: 9 and SEQ ID NO: 6,
respectively) as primers. Comparing the nucleotide sequence of the human OBM cDNA
fragment with the corresponding part of the mouse OBM cDNA, they share a homology of
80.7%.

(4) Screening for Full Length Human OBM cDNA by Hybridization With Human OBM cDNA Fragments With a Length of About 690 bp as Probes

35

The human OBM cDNA fragments, with a length of about 690 bp, purified in Example 19-(3) were labeled with [$\alpha^{32}\text{P}$] dCTP by use of a MEGA PRIME DNA labeling kit (Amersham Co., Ltd.), and full length human OBM cDNA was screened. As an object to be screened, a Human Lymph Node 5'-STRETCH PLUS cDNA library (Clontech Co., Ltd., USA) was used. In accordance with a protocol issued by the company, after *Escherichia coli* C600 Hfl was infected with recombinant phage at 37°C for 15 minutes, the *Escherichia coli* was added to an LB agar medium (1% trypton, 0.5% yeast extract, 1% NaCl, 0.7% agar) heated at 45°C and poured onto an LB agar medium plate containing 1.5% agar. After overnight culturing at 37°C, HYBOND® N (Amersham Co., Ltd.) was brought into intimate contact with the plate having plaques formed thereon for about 3 minutes. Then, this filter was subjected to an alkaline denaturation treatment in accordance with a commonly used method, neutralized, and immersed in a 2X SSC solution. The DNA was fixed on the filter by UV CROSSLINK (Stratagene Co., Ltd.). The obtained filter was immersed in a Rapid-hyb buffer (Amersham Co., Ltd.) and pretreated at 65°C for 15 minutes. Thereafter, the filter was transferred into the above buffer containing the above heat denatured human OBM cDNA fragments (about 690 bp, 5×10^5 cpm/ml) and allowed to hybridize at 65°C overnight. After the reaction, the filter was washed with 0.1%-SDS-containing 2X SSC once, with 1X SSC once and with 0.1X SSC once in turn at 65°C for 15 minutes. The obtained positive clones were screened two more times so as to purify the clones. A clone having about 2.2 kb of insert was selected out of these and used in the following experiment. The purified phage was named λ hOBM. From the purified λ hOBM, about 10 μg of DNA was obtained in accordance with a protocol of a QIAGEN® Lambda kit (QIAGEN Co., Ltd.). After this DNA was cleaved with a restriction enzyme SalI, about 2.2 kb of hOBM insert cDNA was separated by agarose electrophoresis. The DNA fragment, purified by use of a QIAEX® gel extraction kit (QIAGEN Co., Ltd.), was cleaved with restriction enzyme SalI in advance and then inserted into dephosphorylated plasmid pUC19 (MBI Co., Ltd.) by use of a DNA ligation kit ver. 2 (Takara Shuzo Co., Ltd.). *Escherichia coli* DH5 α (Gibco BRL Co., Ltd.) was transformed by use of the pUC19 containing obtained DNA fragment. The obtained transformant was named pUC19hOBM. After proliferating the transformant, about 2.2 kb of human-OBM cDNA-inserted plasmids were purified therefrom in accordance with a commonly used method.

(5) Determination of the Nucleotide Sequence of cDNA Encoding the Full Length Amino Acid Sequence of Human OBM

The nucleotide sequence of the human OBM cDNA obtained in Example 19-(4) was determined by use of a Taq Dideoxy Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.). That is, the nucleotide sequence of the inserted fragment was determined by use

of pUC19hOBM as a template. M13 Primer M3, M13 Primer RV (TAKARA SHUZO CO., LTD.), and a synthetic primer human OBM #8 designed based on the nucleotide sequence of the human OBM cDNA fragment (about 690 bp) were used as primers for determining the nucleotide sequence of the inserted fragment DNA of pUC19. The sequences of the primers, M13 Primer M3 and M13 Primer RV, are shown in SEQ ID NO: 4 and SEQ ID NO: 5, respectively. The amino acid sequence of human OBM estimated from the nucleotide sequence of the human OBM cDNA is shown in SEQ ID NO: 11, and the nucleotide sequence of the human OBM cDNA is shown in SEQ ID NO: 12.

The obtained plasmid containing the human OBM cDNA and the obtained *Escherichia coli* transformed by pUC19hOBM were deposited with the National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry with the deposition number FERM BP-6058 on August 13, 1997.

[Example 20]

¹²⁵I Labeling of OCIF and Quantitative Determination of ¹²⁵I-Labeled OCIF by ELISA

OCIF was ¹²⁵I-labeled in accordance with Iodogen method. 20 µl of 2.5 mg/ml Iodogen-chloroform solution was transferred to a 1.5 ml Eppendorf tube, and chloroform was evaporated at 40°C so as to prepare an Iodogen-coated tube. After the tube was washed with 400 µl of 0.5 M sodium phosphate buffer (Na-Pi; pH 7.0) three times, 5 µl of 0.5 M Na-Pi with a pH of 7.0 was added. Immediately after 1.3 µl (18.5 MBq) of Na-¹²⁵I solution (Amersham Co., Ltd., NEZ-033H) was added to the tube, 10 µl of 1 mg/ml OCIF solution (monomer type or dimer type) was added. The resulting solution was mixed by means of a vortex mixer and left to stand at room temperature for 30 seconds. The solution was transferred to a tube containing 80 µl of 0.5 M Na-Pi solution (pH 7.0), which contained 10 mg/ml potassium iodide and 5 µl of phosphate buffered saline containing 5% bovine serum albumin (BSA-PBS), and mixed. The solution was added to a spin column (1 ml, G-25 Sephadex® fine, Pharmacia Co., Ltd.) equilibrated with BSA-PBS and centrifuged at 2,000 rpm for 5 minutes. After 400 µl of BSA-PBS was added to a fraction eluted from the column and the fraction was mixed, 2 µl of the each fraction was sampled, and the radioactivity of the sample was measured by means of a gamma counter. The radiochemical purity of the prepared ¹²⁵I labeled OCIF solution was determined by measuring the radioactivity of a fraction precipitated by addition 10% trichloroacetic acid (TCA).

The OCIF biological activity of the ¹²⁵I labeled OCIF was measured in accordance with a method described in WO 96/26217. Further, the concentration of ¹²⁵I labeled OCIF was measured by ELISA in the following manner. That is, 100 µl of 50 mM

NaHCO₃ (pH 9.6), having 2 µg/ml of rabbit anti-OCIF polyclonal antibody described in WO 96/26217 dissolved therein, was added to each well of 96-well immunoplate (Nunc Co., Ltd., MaxiSorp) and left to stand at 4°C overnight. After this solution was discarded, 200 µl of mix-solution of BLOCKACE (Snow Brand Milk Products Co., Ltd.) and a phosphate buffered saline (mixing ratio = 25:75) (B-PBS) was added to each well and then left to stand at room temperature for 2 hours. After the solution was discarded, each well was washed with a phosphate buffered saline containing 0.01% Polysorbate 80 (P-PBS) three times. Thereafter, 100 µl of B-PBS containing a ¹²⁵I labeled OCIF sample or OCIF reference standard was added to each well and left to stand at room temperature for 2 hours. After the solution was discarded, each well was washed with 200 µl of P-PBS six times. Then, 100 µl of diluted solution of peroxidase-labeled anti-OCIF rabbit polyclonal antibody in B-PBS was added to each well and left to stand at room temperature for 2 hours. After the solution was discarded, each well was washed with 200 µl of P-PBS six times. Then, 100 µl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well and then left to stand at room temperature for 2 to 3 minutes. Thereafter, 100 µl of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at 450 nm was measured by means of a microplate reader. The concentration of the ¹²⁵I labeled OCIF was determined from a calibration curve prepared by use of the OCIF reference standard.

[Example 21]

Expression of Protein Encoded by the cDNA of the Present Invention

(1) Construction of hOBM Expression Vector for Animal Cell

pUChOBM was cleaved with restriction enzyme Sall, and about 2.2 kb DNA fragments were purified by 1% agarose gel electrophoresis and blunt-ended with DNA Blunting Kit (Takara Shuzo Co., Ltd.) (the resulting DNA fragment with smoothed terminals is called "smoothed hOBM cDNA fragment"). Expression plasmid pcDL-SR α296 (Molecular and Cellular Biology, Vol. 8, pp. 466 to 472 (1988)) was cleaved with a restriction enzyme EcoRI, and blunt-ended with the blunting kit. The resulted expression plasmid was bound to the smoothed hOBM cDNA fragment by use of a DNA ligation kit ver. 2. Using the ligation reaction solution, *Escherichia coli* DHα was transformed. From the obtained ampicillin-resistant transformant, a clone, having a phOBM plasmid in which hOBM cDNA inserted with forward direction for transcription direction of SRα promoter, was selected by analysis of DNA map obtained by restriction enzyme cleavage and determination of DNA sequences. The obtained clone was named DH5α/phOBM.

(2) Expression of Human OBM in COS-7 Cell

E. coli, DH5α/phOBM, was cultured and the plasmid phOBM was purified with

QIA® Filter Plasmid Midi Kit (QIAGEN Co., Ltd.). The phOBM was transfected into COS-7 cells in each well of 6 well plate by use of lipofectamine, and the cells were cultured in DMEM containing 10% fetal bovine serum for 2 days. The medium was replaced with cysteine/methionine-free DMEM (Dainippon Pharmaceutical Co., Ltd.) containing 5% dialyzed fetal bovine serum (88 µl/well), and the cells were cultured for another 15 minutes. Then, 14 µl of Express Protein Labeling Mix (NEN Co., Ltd., 10 mCi/ml) was added. After the cells were cultured for 4 hours, 200 µl of DMEM containing 10% fetal bovine serum was added, and the cells were cultured for 1 hour. After the cells were washed with PBS twice, 0.5 ml of TSA buffer (10 mM Tris-HCl (pH 8.0) containing 0.14 M NaCl and 0.025% NaN₃) containing 1% Triton X-100, 1% bovine hemoglobin, 10 µg/ml leupeptin, 0.2 TIU/ml aprotinin and 1 mM PMSF was added, and the cells were left to stand on ice for 1 hour. After the cells were crushed by pipetting, the resulting lysate was centrifuged at 4°C and 3,000 X g for 10 minutes so as to obtain a supernatant. 200 µl of dilution buffer (TSA buffer containing 0.1% Triton X-100, 0.1% bovine hemoglobin, 10 µg/ml leupeptin, 0.2 TIU/ml aprotinin and 1 mM PMSF) was added to 100 µl of the supernatant, and the resulting supernatant was shaken together with Protein A Sepharose® (50 µl) at 4°C for 1 hour. Thereafter, the solution was centrifuged at 4°C, 1,500 X g for 1 minute so as to collect a supernatant. Thereby, a protein non-specifically binding the Protein A Sepharose® was removed. OCIF (1 µg) was added to the supernatant, and the resulting supernatant was shaken at 4°C for 1 hour so as to bind OBM and OCIF together. Then, an anti-OCIF rabbit polyclonal antibody (50 µg) was added, and the resulting solution was shaken at 4°C for 1 hour. Then, Protein A Sepharose® (10 µl) was added to the solution and the solution was then shaken at 4°C for 1 hour. The solution was centrifuged at 4°C, 1,500 X g for 1 minute so as to collect a precipitated fraction. The precipitate resulting from the centrifugation was washed with the dilution buffer twice, with a bovine hemoglobin free dilution buffer twice, with a TSA buffer once, and with 50 mM Tris-HCl (pH 6.5) once. After washing, an SDS buffer (0.125 M Tris-HCl, 4% dodecyl sodium sulfate, 20% glycerol, 0.002% bromophenol blue, pH 6.8) containing 10% β-mercaptoethanol was added to the precipitate. The precipitate was heated at 100°C for 5 minutes, and it was subjected to SDS-PAGE (12.5% polyacrylamide gel, Daiichi Kagaku Co., Ltd.). The gel was fixed and dried in accordance with a commonly used method, and the signals of isotopes from the fixed gel were amplified by Amplify® (Amersham Co., Ltd.). The fixed gel was exposed to BioMax® MR Film (Kodak Co., Ltd.) at -80°C. The results are shown in Fig. 8. As a result, it was revealed that the molecular weight of protein encoded by the cDNA of the present invention was about 40,000.

[Example 22]

Binding of Protein Encoded by the cDNA of the Present Invention to OCIF

In the same manner as in Example 21-(2), the purified phOBM was transfected into COS-7 cells in each well of a 24 well plate by the use of lipofectamine, and the cells were cultured for 2 or 3 days. Then, the cells were washed with serum-free DMEM, and 200 μ l of medium for the binding assay (serum-free DMEM containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.1 mg/ml heparin and 0.2% NaN₃), containing 20 ng/ml of ¹²⁵I labeled OCIF was added to some wells. In addition, to other wells, 200 μ l of the medium for binding assay, containing 8 μ g/ml of unlabeled OCIF in addition to 20 ng/ml of the ¹²⁵I labeled OCIF, was added so as to conduct following experiments. After culture in a CO₂ incubator (5% CO₂) at 37°C for 1 hour, the cells were washed twice with 500 μ l of phosphate buffered saline containing 0.1 mg/ml heparin. After washing, 500 μ l of 0.1 N NaOH solution was added to each well, and the wells were then left to stand at room temperature for 10 minutes so as to dissolve the cells. The amount of ¹²⁵I in each well was measured by means of a gamma counter. As a result, it was confirmed that the ¹²⁵I labeled OCIF bound only to a cell transfected with phOBM as shown in Fig. 19. Further, it was also confirmed that the binding was significantly inhibited by addition of a 400-fold concentration of unlabeled OCIF (8 μ g/ml). From these results, it was revealed that a human OBM protein, coded for by a cDNA on phOBM, specifically bound to OCIF on the surface of a COS-7 cell.

[Example 23]

Crosslinking Experiment of ¹²⁵I Labeled OCIF to Protein Encoded by the cDNA of the Present Invention

To further analyze the characteristics of the protein encoded by the cDNA of the present invention, crosslinking of ¹²⁵I labeled monomer type OCIF with the protein encoded by the cDNA of the present invention was conducted. That is, after expression vectors phOBM were prepared and transfected into COS-7 cells in accordance with the methods described in Examples 21-(1) and (2), 200 μ l of the medium for the binding assay containing the ¹²⁵I labeled OCIF (25 ng/ml) was added to some wells. In addition, the medium for the binding assay, containing unlabeled OCIF of a 400-fold concentration in addition to the ¹²⁵I labeled OCIF, was added to other wells. The cells were cultured in a CO₂ incubator (5% CO₂) at 37°C for 1 hour, and the cells were washed twice with 500 μ l of phosphate buffered saline containing 0.1 mg/ml of heparin. To these cells, 500 μ l of phosphate buffered saline containing 100 μ g/ml of crosslinking agent DSS (Disuccinimidyl suberate, Pierce Co., Ltd.) was added, and the cells incubated at 0°C for 10 minutes for reaction. After the cells in these wells were washed twice with 1 ml of phosphate buffered saline cooled to 0°C, 100 μ l of 20

mM Hepes buffer containing 1% Triton X-100 (Wako Pure Chemical Industries, Ltd.), 2 mM PMSF (phenylmethylsulfonyl fluoride, Sigma Co., Ltd.), 10 μ M pepstatin (Wako Pure Chemical Industries, Ltd.), 10 μ M leupeptin (Wako Pure Chemical Industries, Ltd.), 10 μ M antipain (Wako Pure Chemical Industries, Ltd.) and 2 mM EDTA (Wako Pure Chemical Industries, Ltd.) were added to these cells, and the wells were left to stand at room temperature for 30 minute so as to lyse the cells. After 15 μ l of these samples were treated with SDS under nonreducing conditions in accordance with a commonly used method, the samples were subjected to electrophoresis with a gel for SDS electrophoresis (4 to 20% polyacrylamide gradient, Daiichi Kagaku Co., Ltd.). After the electrophoresis, the gel was dried and exposed to BioMax® MS Film (Kodak Co., Ltd.) with BioMax® MS Intensifying Amplifying Screen (Kodak Co., Ltd.) at -80°C for 24 hours. The exposed films were developed in accordance with a commonly used method. As a result, a protein band having a molecular weight of about 90,000 to 110,000 was detected as shown in Fig. 20 by crosslinking between 125 I labeled monomer type OCIF and the protein encoded by the cDNA of the present invention.

[Example 24]

Expression of Secretory-Type Human OBM

(1) Construction of Secretory-Type Human OBM Expressing Plasmid

A PCR reaction was carried out by use of human OBM SF (SEQ ID NO: 13) and mouse OBM #8 (SEQ ID NO: 6) as primers and pUC19hOBM as a template. After the product was purified by agarose gel electrophoresis, it was cleaved with restriction enzymes SpI and HindIII and then purified by agarose gel electrophoresis so as to obtain 0.27 kb fragment. A fragment of hOBM cDNA which was cleaved at only one site of restriction enzyme DraI by partial cleavage of human OBM cDNA therewith, and purified by agarose gel electrophoresis, and the purified fragment was further cleaved with a restriction enzyme HindIII. 0.53 kb of DraI/HindIII fragment was purified by agarose gel electrophoresis, and the purified fragment and the SpI/HindIII fragment (0.27 kb) of the above PCR product together with an SpI/EcoRV fragment (5.2 kb) of pSec TagA (Invitrogen Co., Ltd.) were subjected to ligation by use of a ligation kit ver. 2 (TAKARA SHUZO CO., LTD.), and *Escherichia coli* DH5 α were transformed by use of the reaction product of ligation. Plasmids were purified from the obtained ampicillin-resistant clone by alkaline SDS method and cleaved by restriction enzymes so as to select a plasmid having 0.27 kb and 0.53 kb of fragments inserted in pSec TagA. The plasmid was subjected to sequencing by use of a Taq Dideoxy Terminator Cycle Sequencing FS Kit (Perkin Elmer Co., Ltd.), thereby confirming that the plasmid had sequences encoding secretory-type human OBM. After the plasmid was cleaved by restriction enzymes NheI and XhoI, a fragment (0.8 kb) corresponding to

secretory-type human OBM cDNA was collected by agarose gel electrophoresis. This fragment was inserted into an *NheI/XhoI* fragment (10.4 kb) of an expression vector pCEP4 (Invitrogen Co., Ltd.) by use of the ligation kit, and *Escherichia coli* DH5 α were transformed by use of the reaction product of the ligation. Plasmids were purified from the obtained
5 ampicillin-resistant clones by alkaline SDS method and cleaved by restriction enzymes so as to select a *Escherichia coli* clone having a secretory-type human OBM expression plasmid (pCEPshOBM) with a target structure. The *Escherichia coli* clone having the pCEPshOBM was cultured, and the pCEPshOBM was purified by use of QIA® Filter Plasmid Midi Kit (QIAGEN Co., Ltd.).

10 (2) Expression of Secretory-Type OBM

293-EBNA cells were suspended in IMDM containing 10% FCS (IMDM-10%FCS), seeded in a collagen-coated 24 well plate (Sumitomo Bakelite Co., Ltd.) in an amount of 2×10^5 cells/2 ml/well, and cultured overnight. To the cells, 1 μ g of pCEPshOBM or pCEP4 was transfected by use of 4 μ l of lipofectamine (Gibco Co., Ltd.),
15 and the cells were cultured for another 2 days in 0.5 ml of serum-free IMDM or IMDM-10%FCS, thereby collecting a conditioned medium. Expression of secretory-type human OBM in the conditioned medium was confirmed in the following manner. That is, sodium hydrogen carbonate was added to the conditioned medium to a final concentration of 0.1 M and left to stand at 4°C overnight, and the human OBM in the conditioned medium was solid-
20 phased in a 96 well plate. BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution diluted 4 times with PBS (B-PBS) was added to each well and the plate was left to stand at room temperature for 2 hours to cause blocking. 3-100 ng/ml of OCIF diluted with B-PBS was added to the wells and left to stand at 37°C for 2 hours. After the plate was washed with PBS containing 0.05% Polysorbate 20 (P-PBS), 100 μ l of peroxidase labeled anti-OCIF
25 antibody described in WO 96/26217 diluted with B-PBS was added to each well and left to stand at 37°C for 2 hours. After each well was washed with P-PBS six times, 100 μ l of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well and then left to stand at room temperature for about 10 minutes. Thereafter, 100 μ l of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at
30 450 nm was measured by means of a microplate reader. The results are shown in Fig. 21. In the plate having the solid-phased conditioned medium of the cells transfected with the pCEPshOBM, absorption at 450 nm increased depending on the concentration of the OCIF added. Meanwhile, in the case where the conditioned medium of the cells transfected only with the vector pCEP4 was solid-phased, no increase in absorption was seen. Further, Fig. 22
35 shows the results of an experiment in which the proportion of the conditioned medium used

for solid phasing was varied within a range of 5 to 90% and a certain concentration of OCIF (50 ng/ml) was added. In the plate having the solid-phased conditioned medium of the cells transfected with the pCEPshOBM, absorption at 450 nm increased along with an increase in the proportion of the conditioned medium added. Meanwhile, in the plate having the solid-phased conditioned medium of the cells transfected with the vector pCEP4, no increase in absorption was observed. From these results, it was confirmed that secretory-type human OBM was expressed in the conditioned medium of the cells transfected with the pCEPshOBM.

[Example 25]

Expression of Thioredoxin-Human OBM Fusion Protein (Trx-hOBM)

(1) Construction of Thioredoxin-Human OBM Fusion Protein (Trx-hOBM) Expression Vector

10 µl of 10X ExTaq buffer (Takara Shuzo Co., Ltd.), 8 µl of 10 mM dNTP (Takara Shuzo Co., Ltd.), 77.5 µl of sterilized distilled water, 2 µl of pUC19hOBM aqueous solution (10 ng/µl), 1 µl of primer mouse OBM #3 (SEQ ID NO: 9) (100 pmol/µl), 1 µl of primer hOBM SalR2 (SEQ ID NO: 14) (100 pmol/µl) and 0.5 µl of ExTaq (5µ/µl) (Takara Shuzo Co., Ltd.) were mixed together in a microcentrifuge tube so as to cause a PCR reaction. After the reaction consisting of 95°C for 5 minutes, 50°C for 1 second, 55°C for 1 minute, 74°C for 1 second and 72°C for 5 minutes, the cycle reaction consisting of at 96°C for 1 minute, 50°C for 1 second, 55°C for 1 minute, 74°C for 1 second and 72°C for 3 minutes, was repeated 25 times. An approximately 750 bp DNA fragment was purified from the whole reaction solution. After the purified DNA fragment (whole) was cleaved with restriction enzymes SalI (TAKARA SHUZO CO., LTD.) and BspHI (NEW ENGLAND BILABS CO., LTD.), 1% agarose gel electrophoretic migration was carried out so as to purify an approximately 320 bp DNA fragment (fragment 1) and dissolve the fragment in 20 µl of sterilized distilled water. Similarly, an approximately 450 bp DNA fragment (fragment 2) which is a cleaved product of 4 µg of pUC19hOBM described in Example 19-(3) by a restriction enzyme BamHI and BspHI (TAKARA SHUZO CO., LTD.) and about 3.6 kb of DNA fragment (fragment 3) which is a cleaved product of 2 µg of pTrXFus (Invitrogen Co., Ltd.) by a restriction enzyme BamHI and SalI (TAKARA SHUZO CO., LTD.) were purified and then dissolved in 20 µl of sterilized distilled water. To purify the DNA fragments, a QIAEXR II gel extraction kit was used. Fragment 1, 2 and 3 were combined by use of a DNA ligation kit ver. 2 (TAKARA SHUZO CO., LTD.) by keeping them at 16°C for 2.5 hours. *Escherichia coli* GI724 strain (Invitrogen Co., Ltd.) was transformed using the ligation reaction solution, in accordance with a method described in an instruction manual

attached to a ThioFusion Expression System (Invitrogen Co., Ltd.). From the obtained ampicillin-resistant transformants, a clone, having a plasmid in which an hOBM cDNA fragment was bound to a thioredoxin gene in the same reading frame, was selected by analysis of DNA mapping obtained by restriction enzyme cleavage and determination of DNA sequences. The obtained strain was named GI724/pTrxhOBM25.

(2) Expression of Trx-OBM in *Escherichia coli*

A GI724/pTrxhOBM strain and a GI724 strain transformed with pTrxFus (GI724/pTrxFus) were cultured shaking at 37°C for 6 hours in 2 ml of RMG-Amp medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 2% casamino acid, 1% glycerol, 1 mM MgCl₂, 100 µg/ml ampicillin, pH 7.4). 0.5 ml of the culture suspension was added to 50 ml of Induction medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 0.2% casamino acid, 0.5% glucose, 1 mM MgCl₂, 100 µg/ml ampicillin, pH 7.4) and cultured shaking at 30°C. L-tryptophan was added so as to achieve a final concentration of 0.1 mg/ml when the value at OD_{600 nm} became about 0.5, and the cells were further shaking-cultured at 30°C for another 6 hours. The culture suspension was centrifuged at 3,000 X g so as to collect cells, and then the collected cell was suspended in 12.5 ml of PBS. The suspension was subjected to an ultrasonic generator (Ultrasonics Co., Ltd.) so as to crush the cells. Then the sample was centrifuged at 7,000 X g for 30 minutes so as to collect a soluble protein fraction as a supernatant. 10 µl of the solution fraction was subjected to SDS-PAGE (10% polyacrylamide) under reducing conditions. As a result, shown in Fig. 23, a protein band having a molecular weight of about 40,000, which could not be seen in the soluble protein fraction of GI724/pTrxFus, was detected in the soluble protein fraction of GI724/pTrxOBM. From the above results, it was confirmed that a thioredoxin-human OBM fusion protein (Trx-OBM) was expressed in the *Escherichia coli* clone.

(3) Binding Ability of Trx-hOBM *Escherichia coli* to OCIF

It was confirmed by the following experiment that the expressed Trx-hOBM bound to OCIF. That is, 100 µl of anti-thioredoxin antibody (Invitrogen Co., Ltd.) diluted to be 1/5,000 with 10 mM sodium hydrogen carbonate aqueous solution was added to each well of a 96 well immunoplate (Nunc Co., Ltd.), and the plate was left to stand at 4°C overnight. After the solution in each cell was discarded, 200 µl of a solution obtained by diluting BLOCKACE (Snow Brand Milk Products Co., Ltd.) to be 1/2 with PBS (BA-PBS) was added to each well, and then the plate was left to stand at room temperature for 1 hour. After the solution was discarded, each well was washed with P-PBS three times. 100 µl of the GI724/pTrxOBM-derived soluble protein fraction solution diluted stepwise with BA-PBS, and 100 µl of the GI724/pTrxFus-derived soluble protein fraction solution diluted stepwise

with BA-PBS, were added to each well and the plate was left to stand at room temperature for 2 hours. After each well was washed with P-PBS three times, 100 µl of OCIF (100 ng/ml) diluted with BA-PBS, was added to each well and the plate was left to stand at room temperature for 2 hours. After each well was washed with P-PBS three times, 100 µl of peroxidase-labeled anti-OCIF antibody described in WO 96/26217, diluted to be 1/2,000 with BA-PBS, was added to each well, and the plate was left to stand at room temperature for 2 hours. After each well was washed with P-PBS six times, 100 µl of TMB solution was added to each well, and then the plate was left to stand at room temperature for about 10 minutes. Thereafter, 100 µl of Stopping Reagent was added to each well. The absorbance of each well at 450 nm was measured by means of a microplate reader. The results are shown in Fig. 24. No difference was observed between absorbance resulted in the presence and absence of the GI724/pTrxFus-derived soluble protein fraction solution and, while with the GI724/pTrxhOBM-derived soluble protein fraction solution, the absorbance increased depending on an increase in the concentration of the GI724/pTrxOBM derived soluble protein fraction solution. Further, Fig. 25 shows the results of an experiment in which the dilution rate of the soluble protein fraction solution to be added was kept constant (1% concentration) and OCIF (0-100 ng/ml) diluted stepwise with BA-PBS was added. For the GI724/pTrxFus-derived soluble protein fraction solution, absorbance remained low regardless of the concentration of OCIF, while for the GI724/pTrxhOBM-derived soluble protein fraction solution, absorbance increased in OCIF concentration-dependent manner. It was confirmed from this result that Trx-hOBM produced in GI724/pTrxhOBM had an ability to bind OCIF.

(4) Large Scale Culture of *Escherichia coli* Producing Trx-hOBM

GI724/pTrxhOBM was spread on an RMG-Amp agar medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 2% casamino acid, 1.5% agar, pH 7.4) with a platinum loop and cultured at 30°C overnight. The cells were suspended in 10 ml of Induction medium, and every 5 ml of the suspension was added to each of two conical flasks of 2L volume containing 500 ml of Induction medium, and the flasks were shaking-cultured at 30°C. L-tryptophan was added so as to achieve a final concentration of 0.1 mg/ml when absorbance at OD_{600 nm} became about 0.5, and the shaking culture at 30°C was continued for another 6 hours. The culture suspension was centrifuged at 3,000 X g for 20 minutes so as to collect cells and the collected cells were then suspended in 160 ml of PBS. The suspension was subjected to ultrasocination (Ultrasonics Co., Ltd.) so as to crush the cells, and the cell lysate was then centrifuged at 7,000 X g for 30 minutes so as to collect a soluble protein fraction as a supernatant.

(5) Preparation of OCIF-Immobilized Affinity Column

2 g of TSKgel AF-Tresyl TOYOPAL 650 (TOSO CO., LTD.) and 40 ml of 1.0 M potassium phosphate buffer (pH 7.5) containing 35.0 mg of recombinant OCIF, prepared by a method described in WO 96/26217, were mixed together and gently shaken at 4°C overnight so as to cause a coupling reaction. To inactivate excessive active residue, after a supernatant was removed by centrifugation, 40 ml of 0.1 M Tris-HCl buffer (pH 7.5) was added to a precipitated carrier, and the mixture was gently shaken at room temperature for 1 hour. After 0.1 M glycine-HCl buffer containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH 3.3) and a 0.1 M sodium citrate buffer containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH 2.0) were passed through a column (in which the obtained gel was packed) so as to wash it, the column was washed twice with 10 mM sodium phosphate buffer containing 0.01% Polysorbate 80 (pH 7.4) so as to equilibrate it.

(6) Purification of Trx-hOBM by OCIF-Immobilized Affinity Column

Purification of Trx-hOBM was carried out at 4°C unless otherwise stated. After the above-mentioned OCIF-immobilized affinity carrier (10 ml) and the above-mentioned soluble protein fraction solution (120 ml) described in Example 25-(4) were mixed together, the mixture was gently shaken at 4°C overnight in four 50 ml centrifuge tubes by use of a rotor. The carrier in the mixture was filled an EconoColumn (internal diameter: 1.5 cm, length: 15 cm, Bio-Rad Co., Ltd.). 300 ml of PBS containing 0.01% Polysorbate 80, 100 ml of 10 mM phosphate buffer containing 0.01% Polysorbate 80 and 2.0 M NaCl (pH 7.0), and 100 ml of 0.1 M glycine-HCl buffer containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH 3.3) were passed through the column, in turn, so as to wash the column. Then, 0.1 M sodium citrate buffer containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH 2.0) was passed through the column so as to elute proteins adsorbed to the column. 5 ml eluates were fractionated. To the fractions, 10% volume of 2M Tris solution (pH 8.0) was added so as to immediately neutralize the fractions. The presence or absence of Trx-hOBM in the each fraction of the eluate was examined in accordance with the method described in Example 25-(3). Fractions containing Trx-hOBM were collected and purified further.

(7) Purification of Trx-hOBM by Gel Filtration

About 25 ml of the Trx-hOBM fraction described in Example 25-(6) was concentrated using a centrifuge to about 0.5 ml by use of a Centriplus R10 and a Centricon R10 (Amicon Co., Ltd.). The concentrated sample was subjected to a Superose R12 HR 10/30 column (1.0 X 30 cm, Pharmacia Co., Ltd.) equilibrated in advance with PBS containing 0.01% Polysorbate 80. The column was developed at a flow rate of 0.25 ml/min by using PBS containing 0.01% Polysorbate 80 as a mobile phase so as to fractionate every 0.25 ml of eluates from the column. Trx-OBM in the fractions was detected by the method described in Example 25-(3) and SDS-PAGE. Fractions containing purified Trx-hOBM were

collected so as to measure the protein concentration of Trx-OBM. The protein concentration was measured with DC-protein assay kit (Bio-Rad Co., Ltd.) using bovine serum albumin as a reference standard.

[Example 26]

5 Osteoclastogenesis Inducing Activity of OBM

phOBM and pcDL-SR α 296 were transfected into COS-7 cells by use of lipofectamine (Gibco Co., Ltd.), respectively. After the cells were cultured in DMEM containing 10% FCS for 1 day, they were trypsinized and seeded in a 24-well plate, in which glass cover slips (15 mm round, Matsunami Co., Ltd.) were seated, at a concentration of 5X
10 10^4 cells/well and then cultured for another two days. The culture plate was washed with PBS once and then PBS containing 1% paraformaldehyde was added, and the cells were incubated at room temperature for 8 minutes so as to fix the cells on the glass cover slips. After the plate with fixed cells was washed with PBS six times, 700 μ l of mouse spleen cells suspended in α -MEM (containing 10^{-8} M activated vitamin D₃, 10^{-7} M dexamethasone and
15 10% fetal bovine serum) in an amount of 1×10^6 cells/ml were added to each well. A MILLICELL® PCF (Millipore Co., Ltd.) was set on each well, and 700 μ l of ST2 cells, suspended in the above medium in a concentration of 4×10^4 cells/ml were added to the MILLICELL® PCF and cultured at 37°C for 6 days. After that, the MILLICELL® PCF was removed and the plate was washed with PBS once. Then, the cells were fixed for a minute by
20 an acetone-ethanol solution (50:50), and cells, having tartaric acid resistant acid phosphatase activity (TRAP activity), which is specific marker of osteoclast, were stained by use of a leukocyte acid phosphatase kit (Sigma Co., Ltd.). Using a microscope, cells having TRAP activity were not detected in the wells having COS-7 cells transfected with the pcDL-SR α 296, while 65 ± 18 ($n = 3$, average \pm standard deviation) of TRAP positive cells were
25 observed in the wells having cells transfected with phOBM. Further, it was also confirmed that these TRAP positive cells expressed calcitonin receptors, since the cells showed specific binding to 125 I-labeled salmon calcitonin (Amersham Co., Ltd.). From these results, it was revealed that human OBM, a protein encoded by the cDNA of the present invention, had an activity to promote osteoclast formation.

30 [Example 27]

Osteoclastogenesis Promoting Activities of Trx-hOBM and Secretory-Type Human OBM

Mouse spleen cells were suspended in α -MEM containing 10^{-8} M activated vitamin D₃, 10^{-7} M dexamethasone and 10% fetal bovine serum at a concentration of 2×10^6
35 cells/ml, and 350 μ l of the suspension was added to each well of a 24 well plate. After 350

μl of a solution prepared by diluting purified Trx-OBM,(40 ng/ml) with the above medium, 350 μl of a solution prepared by diluting a conditioned medium obtained when 293-EBNA cells transduced by pCEPshOBM or pCEP4 were cultured in IMDM-10%FCS to be 1/10 with the above medium, or 350 μl of the above medium alone was added, a MILLICELL® PCF (Millipore Co., Ltd.) was set on each well, and 600 μl of ST2 cell suspension in the above medium at a concentration of 4×10^4 cells/ml were added to the Millicell® PCF. After the cell were cultured for 6 days, the Millicell® PCF was removed, and the plate was washed with PBS once. Then, after the cells were fixed for 1 minute by an acetone-ethanol solution (50:50), cells having tartaric acid resistant acid phosphatase activity (TRAP activity) were stained by use of a leukocyte acid phosphatase kit (Sigma Co., Ltd.). Using a microscope, cells having TRAP activity were not detected in the wells not containing Trx-hOBM, while 115 ± 19 ($n = 3$, average \pm standard deviation) of TRAP positive cells were observed in the wells containing Trx-hOBM. Similarly, cells having TRAP activity were not detected in the wells containing the conditioned medium of pCEP4-transfected 293-EBNA, while 125 ± 23 ($n = 3$, average \pm standard deviation) of TRAP positive cells were observed in the wells containing the conditioned medium of pCEPshOBM-transfected 293-EBNA. Furthermore, it was also confirmed that these TRAP positive cells expressed calcitonin receptors, since the cells showed specific binding to ^{125}I labeled salmon calcitonin (Amersham Co., Ltd.). From these results, it was revealed that Trx-hOBM and secretory-type OBM had an activity to promote osteoclast formation.

[Example 28]

Preparation of Polyclonal Antibody

Mouse sOBM or human sOBM, which was used as an immunizing antigen, was obtained in accordance with the above-mentioned method. That is, mouse sOBM cDNA (cDNA which encodes mouse sOBM (SEQ ID NO: 16) having no membrane binding site and lacking amino acids between the N-terminal end and amino acid 72 of mouse OBM; SEQ ID NO: 18) or human OBM cDNA (cDNA which encodes human sOBM (SEQ ID NO: 17) having no membrane binding site and lacking amino acids region between the N terminal end and amino acid 71 of human OBM; SEQ ID NO: 19), together with a Hind III/EcoRV fragment (5.2 kb) of a pSec TagA expression vector (Invitrogen Co., Ltd.), containing nucleotide sequence coding a signal peptide of κ -chain of immunoglobulin, and an EcoRI/PmaCI fragment (0.32 kb) of OBM cDNA, were subjected to ligation by use of a ligation kit ver. 2 (TAKARA SHUZO CO., LTD.). *Escherichia coli* DH5 α were transformed with the reaction product. Plasmids were purified from the obtained ampicillin-resistant clones by alkaline SDS method and cleaved by restriction enzymes so as to select a plasmid

having 0.6 kb and 0.32 kb of fragments inserted in pSec TagA. As a result of determining the sequences of the plasmid by use of Dye Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.), it was confirmed that the plasmid had sequences encoding mouse or human sOBM. The plasmid was cleaved by restriction enzymes NheI and XhoI and then a fragment (1.0 kb) corresponding to secretory-type OBM cDNA was collected by agarose gel electrophoresis. The fragment was inserted into an NheI/XhoI fragment (10.4 kb) of an expression vector pCEP4 (Invitrogen Co., Ltd.) by use of a ligation kit, and *Escherichia coli* DH5 α were transformed by use of the reaction product. Plasmids were purified from the obtained ampicillin-resistant clones by an alkaline SDS method and cleaved by restriction enzymes and analyzed so as to select a *Escherichia coli* clone having a secretory OBM expression plasmid (pCEP sOBM) with the target structure. The *Echerichia coli* clone having the pCEP sOBM was cultured, and the pCEP sOBM was purified by use of a QIA® Filter Plasmid Midi Kit (QIAGEN Co., Ltd.). Next, 293-EBNA cell was suspended in IMDM containing 10% FCS (IMDM-10%FCS) and seeded in a collagen-coated 24 well plate (Sumitomo Bakelite Co., Ltd.) in an amount of 2×10^5 cells/2 ml/well, and cultured overnight. To the cells, 1 μ g of pCEP sOBM or pCEP4 was transfected by use of 4 μ l of lipofectamine (Gibco Co., Ltd.), and the cells were cultured for another 2 days in 0.5 ml of serum-free IMDM or IMDM-10%FCS, thereby collecting a conditioned medium. The clones with high production of recombinant mouse soluble OBM (msOBM) or human soluble OBM (hsOBM) were screened in the following manner. After sodium hydrogen carbonate was added to the conditioned medium seemed to contain msOBM or hsOBM at a final concentration of 0.1 M, 100 μ l of the conditioned medium was added to each well of 96 well immunoplate (Nunc Co., Ltd.) and the plate was left to stand at 4°C overnight so as to solid-phase the msOBM or hsOBM in the conditioned medium on each well. Then, 200 μ l of BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution diluted to 4 times with PBS (B-PBS) was added to each well of the plate and the plate was left to stand at room temperature for 2 hours. After washing three times with PBS containing 0.1% Polysorbate 20 (P-PBS), 100 μ l of recombinant OCIF (rOCIF) solution diluted stepwise (0-100 ng/ml) with B-PBS was added to each well and the plate was left to stand at 37°C for 2 hours. After washing three times with PBS, 100 μ l of peroxidase labeled anti-OCIF polyclonal antibody (WO 96/26217), diluted with B-PBS, was added to each well and the plate was left to stand at 37°C for 2 hours. After washing six times with P-PBS, 100 μ l of TMB solution (TMB Soluble Agent, High sensitivity, Scytek Co., Ltd.) was added to each well and left to stand at room temperature for about 10 minutes. Thereafter, 100 μ l of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at 450 nm was measured by

means of a microplate reader. In the plate having the solid-phased protein derived from conditioned medium of the clone producing msOBM or hsOBM, the absorbance significantly increased in proportion to the concentration of the OCIF. As for the clones producing msOBM or hsOBM, clones indicating a high rate of increase in the absorbance were selected as highly producing clones thereof. Each of the highly producing clones of msOBM or hsOBM selected in the above mentioned manner were mass-cultured by use of IMDM containing 5% FCS as a medium in 25 T-flasks (T-225). After the cells grew to confluency, 100 ml of fresh medium was added to each T-255 flask and the cells were further cultured for 3 or 4 days, and then a conditioned medium was collected. By repeating this procedure 4 times, 10 liters of the conditioned medium containing msOBM and 10 liters of the conditioned medium containing hsOBM were obtained. About 10 mg of purified msOBM and about 12 mg of purified hsOBM, which were uniform (molecular weight: 32 kDa) in terms of SDS-polyacrylamide electrophoresis, were obtained by carrying out purification on the above-obtained conditioned medium with affinity chromatography using an rOCIF-immobilized column and gel filtration chromatography in accordance with the method described in Examples 25-(6) and (7). The obtained purified samples were used as immunizing antigens. The obtained antigens each were dissolved in phosphate buffered saline (PBS) at a concentration of 200 µg/ml and then the solution was mixed with an equal amount of Freund's complete adjuvant so as to be emulsified. 1 ml of each emulsion was subcutaneously administered to three Japanese white rabbits at intervals of about one week so as to immunize the rabbits. An antibody titer was measured, and when the antibody titer reached a maximum, a booster was carried out. 10 days after the booster, all blood was collected from all the rabbits. Antiserum was diluted to two times with binding buffer for Protein A Sepharose® chromatography (Bio-Rad Co., Ltd.) and then added to a Protein A column equilibrated with the above buffer. After the column was efficiently washed with the above buffer, an anti-sOBM antibody adsorbed to the column was eluted by an elution buffer (Bio-Rad Co., Ltd.) or 0.1 M glycine-HCl buffer (pH 2.9 to 3.0). In order to immediately neutralize the antibody-containing eluate, the eluted solution was fractionated by use of a test tube containing a small amount of 1.0 M Tris-HCl (pH 8.0). The antibody eluate was dialyzed in PBS at 4°C overnight. The amount of protein in the antibody solution was measured in accordance with the Lowry method using bovine IgG as a standard. Thus, the purified immunoglobulin (IgG) containing the polyclonal antibody of the present invention was obtained in an amount of about 10 mg per 1 ml of rabbit antiserum.

[Example 29]

Measurements of OBM and sOBM by ELISA Using Polyclonal Antibody

Sandwich ELISAs, using the rabbit anti-hsOBM polyclonal antibody obtained

in Example 28 as a solid phase antibody and as an enzyme labeled antibody, were constructed. As enzyme labeling, peroxidase (POD) labeling was carried out in accordance with a method of Ishikawa *et al.* (Ishikawa *et al.*: J. Immunoassay, Vol. 4, 209 to 327, 1983). The anti-hsOBM polyclonal antibody obtained in Example 28 was dissolved in a 0.1 M NaHCO₃ solution at a concentration of 2 µg/ml, and 100 µl of the resulting solution was added to each well of 96-well immunoplate (Nunc Co., Ltd.) and the plate was left to stand at room temperature overnight. Then, 200 µl of 50% BLOCKACE (Snow Brand Milk Products Co., Ltd.) was added to each well, and the plate was left to stand at room temperature for 1 hour. Each well was washed with PBS containing 0.1% polysorbate 20 (washing buffer) three times. The purified human OBM, which was expressed in the same manner as in Example 26 and was purified in the same manner as in Example 2, and the purified human sOBM, obtained in Example 28, was diluted stepwise with primary reaction buffer (0.2 M Tris-HCl buffer containing 40% BLOCKACE and 0.1% polysorbate 20, pH 7.2), and 100 µl of each diluent were added to each well. After the plate was left to stand at room temperature for 2 hours, each well was washed with the above washing buffer three times. 100 µl of POD labeled anti-human sOBM polyclonal antibody, diluted 1,000 times with secondary reaction buffer (0.1 M Tris-HCl buffer containing 25% BLOCKACE and 0.1% polysorbate 20, pH 7.2) was added to each well and the plate was left to stand at room temperature for 2 hours. Each well was washed with the washing buffer three times. 100 µl of substrate solution (TMB, ScyTek Co., Ltd.) was added to each well, and the plate was left to stand at room temperature for 10 minutes. 100 µl of reaction stopping solution (Stopping reagent, ScyTek Co., Ltd.) was added to each well so as to stop the enzyme reaction. The absorbance at 450 nm of each well was measured by use of a microplate reader. The results are shown in Fig. 26. The sandwich ELISA, using the rabbit anti-human sOBM polyclonal antibody, almost equally detected both human sOBM (molecular weight: about 32 kDa) and human OBM (molecular weight: about 40 kDa), and measurement sensitivity was about 12.5×10^{-3} pmol/ml (about 500 pg/ml for human OBM, about 400 pg/ml for human sOBM). It was revealed that measurements of mouse sOBM and mouse OBM by ELISA using the rabbit anti-mouse sOBM polyclonal antibody obtained in Example 28 could be made in the same manner as described above, measurement sensitivity in measuring mouse OBM or mouse sOBM was similar with that in human OBM or human sOBM, and a very small amount of mouse sOBM or mouse OBM could be measured.

As described above, since the present anti-human sOBM polyclonal antibody obtained in Example 28 recognized both human sOBM and human OBM as antigen equally, it was named an anti-human OBM/sOBM polyclonal antibody. Meanwhile, since the anti-

mouse sOBM polyclonal antibody obtained in Example 28 recognized both mouse sOBM and mouse OBM as antigen equally, it was named an anti-mouse OBM/sOBM polyclonal antibody.

[Example 30]

5 **Preparation of Monoclonal Antibody**

10 The purified human sOBM obtained in Example 28 was used as an immunizing antigen. The purified human sOBM was dissolved in phosphate buffered saline at a concentration of 10 µg/ml. To the prepared human sOBM solution, an equal amount of Freund's complete adjuvant was added so as to emulsify it. Thereafter, 200 µl of the antigen was administered into the abdominal cavity of each Balb/c mouse at an interval of one week for a total of three times so as to immunize the mice. Then, to a physiological saline solution containing 5 µg/ml of the human sOBM, an equal amount of Freund's incomplete adjuvant was added so as to fully emulsify it, and 200 µl of the emulsion was administered to each of the above Balb/c mice at one week intervals for a total of four times so as to further immunize the mice. After the passage of one week from the fourth additional immunization, 100 µl of phosphate buffered saline solution containing 10 µg/ml of the human sOBM was parenterally administered to each of the Balb/c mice for booster. On the 3rd day after the final immunization, the spleen was removed, and spleen cells were separated and fused with mouse myeloma cells P3x63-AG8.653 in accordance with a known method (Koehler, G. and Milstein, C., Nature, 256, 495 (1975)). After completion of the fusion, the cell suspension was cultured in a HAT medium containing hypoxanthine, aminopterin and thymidine for 10 days. After the myeloma cells perished and hybridomas appeared, the medium was replaced with an HT medium obtained by removing aminopterin from the HAT medium, and the culture was continued.

25 [Example 31].

Selection and Cloning of Hybridoma

 Since the appearance of the hybridoma was seen on the 10th day from the start of the cell fusion and culturing in Example 30, a high affinity antibody recognizing human sOBM and hybridoma producing the antibody were selected in the means of the following improved solid phase ELISA. Further, to select an anti-OBM monoclonal antibody recognizing both of human sOBM and mouse sOBM, the mouse sOBM obtained in Example 27 as well as human sOBM was used as an antigen in the solid phase ELISA. Human sOBM and mouse sOBM each was dissolved in 0.1 M sodium hydrogen carbonate solution at a concentration of 5 µg/ml, and 50 µl of each antigen solution was added to each well of a 96-well immunoplate (Nunc Co., Ltd.), and the plate was left to stand at 4°C overnight so as

to attach the antigens. The antigen solution in each well was discarded, and 200 μ l of 50% BLOCKACE (Snow Brand Milk Products Co., Ltd.) was added to each well. The plate was left to stand at room temperature for 1 hour so as to cause blocking. After each well was washed with a phosphate buffered saline containing 0.1% polysorbate 20, 40 μ l of bovine serum (Hyclone Co., Ltd.) was added to each well. Then, 10 μ l of hybridoma conditioned medium was added to each well and the plate was left to stand under a serum concentration of 80% at room temperature for 2 hours so as to cause reaction. An object of the solid phase ELISA in the presence of 80% serum is to select an antibody capable of binding to a small amount of human sOBM or mouse sOBM even in the presence of protein and a serum-derived immune reaction inhibiting substance in high concentration, that is, to select a hybridoma producing an antibody having high affinity for human sOBM or mouse sOBM. After completion of the reaction at room temperature for 2 hours, the plate was washed with PBS-P, and 50 μ l of diluent of peroxidase labeled anti-mouse IgG (KPL CO., LTD.) diluted to 5,000 times with a physiological saline solution containing 25% BLOCKACE was added to each well, and the plate was left to stand at room temperature for 2 hours so as to cause a reaction. After the plate was washed with PBS-P three times, 50 μ l of substrate solution (TMB, ScyTek Co., Ltd.) was added to each well and left to stand at room temperature for 5 minutes. Then, 50 μ l of a reaction stopping reagent (Stopping Reagent, ScyTek Co., Ltd.) was added so as to terminate the enzyme reaction. The absorbance at 450 nm of each well was measured by use of a microplate reader (IMMUNOREADER NJ2000, Nippon Intermed Co., Ltd.) so as to select a hybridoma producing an antibody which recognizes human sOBM or mouse sOBM. The hybridomas showing particularly high absorbance (OD_{450nm}) were selected and repeatedly cloned 3 to 5 times by a limiting dilution method so as to established hybridomas producing antibody stably. Out of the obtained hybridomas, hybridomas having higher antibody productivity were selected.

[Example 32]

Production and Purification of Monoclonal Antibody

The antibodies obtained in Example 31, that is, the hybridoma producing an antibody which recognizes human sOBM with high affinity and the hybridoma producing the antibody which has a cross-reactivity with mouse sOBM were cultured, and each hybridoma was implanted in the abdominal cavity of a Balb/c-based mouse which had been given pristane (Aldrich Chemical Co., Ltd.) about a week before, in an amount of 1×10^6 cells/mouse. After about 2 or 3 weeks, accumulated ascites was sampled so as to obtain ascites containing the monoclonal antibody recognized human sOBM or the monoclonal antibody recognizing human sOBM and mouse sOBM. Purified monoclonal antibodies were

obtained from the ascites using Protein A column (Pharmacia Co., Ltd.) chromatography in accordance with the method for purifying an anti-OBM/sOBM polyclonal antibody described in Example 28.

[Example 33]

5 Antigenic Specificity of the Monoclonal Antibody

The antigenic specificities of monoclonal antibodies, which specifically recognized human sOBM, and of monoclonal antibodies, having cross-reactivity with human sOBM and mouse sOBM, were examined using human sOBM, intact human OBM having a membrane binding site, mouse sOBM, and intact mouse OBM having a membrane binding site as antigens. Although over 30 types of monoclonal antibodies were obtained, the results of representative monoclonal antibodies are shown in Table 1. As a result, it was revealed that most of anti-human sOBM monoclonal antibodies which specifically recognized human sOBM recognized even intact human OBM having a membrane binding site and did not recognize mouse sOBM and intact mouse OBM having a membrane binding site.

Meanwhile, a few monoclonal antibodies recognizing both of human sOBM and mouse sOBM were also obtained and it was found that these antibodies had cross-reactivity with human OBM and mouse OBM. These results indicate that human OBM and mouse OBM had a common antigen recognition site, i.e., epitope. Since an anti-human sOBM monoclonal antibody prepared by use of human sOBM as an antigen also equally recognized human OBM, which was a membrane binding intact protein, the monoclonal antibody was named an anti-human OBM/sOBM monoclonal antibody.

Table 1

Antibody	Antigen			
	hsOBM	hOBM	msOBM	mOBM
H-OBM 1	+	+	-	-
H-OBM 2	+	+	-	-
H-OBM 3	+	+	-	-
H-OBM 4	+	+	-	-
H-OBM 5	+	+	-	-
H-OBM 6	+	+	-	-
H-OBM 7	+	+	-	-
H-OBM 8	+	+	-	-
H-OBM 9	+	+	+	+
H-OBM 10	+	+	-	-
H-OBM 11	+	+	-	-
H-OBM 12	+	+	-	-
H-OBM 13	+	+	+	+
H-OBM 14	+	+	-	-

(hsOBM: human soluble OBM, hOBM: human membrane binding OBM, msOBM: mouse soluble OBM, mOBM: mouse membrane binding OBM)

[Example 34]

Tests of Class and Subclass of Monoclonal Antibody

The class and subclass of the monoclonal antibody of the present invention were determined by use of the Immunoglobulin Class/Subclass Analytical Kit (Amersham Co., Ltd.). The tests were conducted in accordance with a protocol provided in the kit. The results of representative monoclonal antibodies are shown in Table 2. The majority of anti-human OBM/sOBM monoclonal antibodies had IgG₁, and some antibodies having IgG_{2a} or IgG_{2b} were also found. Further, all of the antibodies had κ chain as a light chain.

Table 2

Antibody	IgG ₁	IgG _{2a}	IgG _{2b}	IgG ₃	IgA	κ
H-OBM 8	-	+	-	-	-	+
H-OBM 9	+	-	-	-	-	+
H-OBM 10	+	-	-	-	-	+
H-OBM 11	+	-	-	-	-	+
H-OBM 12	-	-	+	-	-	+
H-OBM 13	+	-	-	-	-	+
H-OBM 14	+	-	-	-	-	+

[Example 35]

Measurement of Dissociation Constant (K_D value) for the Monoclonal Antibodies

The dissociation constants for monoclonal antibodies were measured in accordance with a known method (Betrand Friguet *et al.*: Journal of Immunological Methods, 77, 305 to 319, 1986). That is, the purified antibody obtained in Example 32 was diluted at 5 ng/ml with 0.4 M Tris-HCl containing 40% BLOCKACE and 0.1% polysorbate 20 (pH 7.4, primary buffer) and an equal amount of diluent of the purified human soluble OBM (hsOBM) obtained in Example 28, prepared with the primary buffer at stepwise-concentration from 6.25 ng/ml to 10 μ g/ml, was added and the solution was left to stand at 4°C for 15 hours so as to bind the monoclonal antibody to hsOBM. After 15 hours, an antibody unbound to hsOBM was measured by solid phase ELISA with solid-phased hsOBM (10 μ g/ml, 100 μ l/well) so as to calculate the dissociation constant of the monoclonal antibody to hsOBM. Further, the affinity for msOBM of monoclonal antibodies, having cross-reactivity with mouse soluble OBM (msOBM) and hsOBM, was also measured by using msOBM in place of hsOBM at the above-mentioned method. Particularly, the results of particular antibodies, which had high affinity for each of the antigens and were useful in enzymatic immunoassay, binding assay and such, are shown in Table 3.

Table 3

Antibody	Subclass	Antigen	Dissociation Constant K _d (M)
H-OBM 1	IgG ₁ (κ)	hsOBM	$1 \times 10^{-11} < K_d < 1 \times 10^{-10}$
H-OBM 4	IgG ₁ (κ)	hsOBM	$1 \times 10^{-11} < K_d < 1 \times 10^{-10}$
H-OBM 9	IgG ₁ (κ)	hsOBM	$1 \times 10^{-9} < K_d < 1 \times 10^{-8}$

H-OBM 9	IgG ₁ (κ)	msOBM	$1 \times 10^{-8} < K_d < 1 \times 10^{-7}$
---------	----------------------	-------	---------------------------------------------

As a result, it was found that H-OBM 1 and H-OBM 4 which were specific antibodies for human soluble OBM (hsOBM) showed a dissociation constant on the order of 10^{-11} M, indicating that they had very high affinity for hsOBM. Meanwhile, the K_d value of H-OBM 9 which was an antibody recognizing both hsOBM and mouse soluble OBM (msOBM) was on the order of 10^{-8} M with respect to msOBM and on the order of 10^{-9} M with respect to hsOBM. Further, regarding H-OBM 13, which was another antibody recognizing both antigens shown in Table 1, the dissociation constants of H-OBM 13 with respect to both antigens were almost identical with those of H-OBM 9, and since both antibodies had the same subclass, a possibility was suggested that they were the same antibody recognizing the same epitope.

[Example 36]

Method for Measuring Human OBM and sOBM by Sandwich ELISA Using Anti-Human OBM/sOBM Monoclonal Antibody

Sandwich ELISA was constructed by use of the two types of high affinity monoclonal antibodies obtained in Example 35, i.e., H-OBM 1 and H-OBM 4, as a solid phase antibody and an enzyme labeled antibody, respectively. Maleimide Activated Peroxidase Kit (Pierce Co., Ltd.) was used for labeling the antibody. H-OBM 1 antibody was dissolved in 0.1 M sodium hydrogen carbonate solution at a concentration of 10 µg/ml, and 100 µl of the resulting solution was added to each well of 96-well immunoplate (Nunc Co., Ltd.). The plate was left to stand at 4°C overnight so as to attach the antibody. After the solution in each well was discarded, 300 µl of 50% BLOCKACE was added to each well, and the plate was left to stand at room temperature for 2 hours so as to cause blocking. After the blocking, the plate was washed with phosphate buffered saline containing 0.1% polysorbate 20 (PBS-P). Human soluble sOBM and human OBM each were dissolved in 0.4 M Tris-HCl (pH 7.4) containing 40% BLOCKACE (Snow Brand Milk Products Co., Ltd.) and 0.1% polysorbate 20 (Wako Pure Chemical Industries, Ltd.) (primary reaction buffer) and diluted so as to prepare test samples with various concentrations. 100 µl of each of test sample, prepared at various concentrations, was added to each well, and the plate was left to stand at room temperature for 2 hours so as to cause a reaction. Thereafter, the plate was washed with PBS-P, and 100 µl of POD labeled H-OBM 4 antibody diluted with 0.2 M Tris-HC (pH 7.4) containing 25% BLOCKACE and 0.1% polysorbate 20 (secondary reaction buffer) was added to each well. The plate was left to stand at room temperature for 2 hours so as to cause a reaction. After the plate was washed with PBS-P, 100 µl of substrate solution (TMB,

ScyTek Co., Ltd.) was added to each well so as to develop color in the wells, and 100 μ l of reaction stopping solution (stopping reagent, ScyTek Co., Ltd.) was added to each well so as to stop the enzyme reaction. The absorbance at 450 nm of each well was measured by use of a microplate reader. The results are shown in Fig. 27.

As a result, it was revealed that the sandwich ELISA constructed by use of the two types of high affinity anti-human OBM/sOBM monoclonal antibodies obtained in Example 35, i.e., H-OBM 1 and H-OBM 4, detected human OBM and human sOBM equally. The measurement sensitivity thereof was about 1.25 to 2.5×10^{-3} pmol/ml (about 50 to 100 pg/ml for human OBM having a molecular weight of about 40 kDa, about 40 to 80 pg/ml for human sOBM having a molecular weight of about 32 kDa), and very small amounts of human OBM and human sOBM could be measured by the ELISA. Hybridomas producing these two types of anti-human OBM/sOBM monoclonal antibodies, H-OBM 1 and H-OBM 4, were named H-OBM1 and H-OBM4, respectively. Further, a hybridoma producing H-OBM 9, the anti-human OBM/sOBM monoclonal antibody which recognized both mouse OBM and mouse sOBM and exhibited osteoclastogenesis inhibitory activity, was named H-OBM9. These hybridomas were deposited with the National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry with deposit numbers FERM BP-6264 (H-OBM1), FERM BP-6265 (H-OBM4) and FERM BP-6266 (H-OBM9) on November 5, 1997.

[Example 37]

Measurements of Mouse OBM and Mouse sOBM Using Anti-Human OBM/sOBM Monoclonal Antibody Recognizing Mouse OBM and Mouse sOBM

Sandwich ELISAs using the anti-human OBM/sOBM monoclonal antibody H-OBM9 recognizing mouse OBM and mouse sOBM and obtained in Examples 33 and 35 as a solid-phased antibody, and using the anti-mouse OBM/sOBM polyclonal antibody obtained in Example 28 as an enzyme labeled antibody, were constructed. Mouse OBM and mouse sOBM were diluted stepwise with the primary reaction buffer in the same manner as in Example 35, and the mouse OBM and mouse sOBM were detected in the same manner as in Example 36. The results are shown in Fig. 28. As a result, it was confirmed that the mouse OBM and mouse sOBM could be detected equally by use of the anti-human OBM/sOBM monoclonal antibody H-OBM 9 which recognized the mouse OBM and mouse sOBM. As shown in the results of Example 35, the antibody H-OBM 9 had a high dissociation constant with respect to the mouse sOBM; in other words, the antibody had relatively low affinity for the mouse sOBM. Thus, the measurement sensitivities of mouse OBM (molecular weight: about 40 kDa) and mouse sOBM (molecular weight: about 32 kDa) by the above ELISA were about 25×10^{-3} pmol/ml (about 1 ng/ml for mouse OBM, about 0.8 ng/ml for mouse sOBM).

[Example 38]

Assay for Osteoclastogenesis Inhibitory Activity of Anti-OBM/sOBM Antibody

It is known that an osteoclast-like cell (OCL) is derived by co-culture of mouse spleen cell and ST2 cell (mouse bone marrow derived interstitial cell) (Endocrinology, 125, 1,805 to 1,813 (1989)). Thus, it was examined whether derivation of OCL was inhibited by addition of an OBM/sOBM antibody to the co-culture. Since mouse OBM was expressed in the co-culture system, antibodies used in this Example were H-OBM 9 and rabbit anti-mouse OBM/sOBM polyclonal antibody recognizing mouse OBM. The OBM antibodies each were diluted stepwise with α MEM containing 10% FCS and added to a 24 well plate (Nunc Co., Ltd.) in an amount of 700 μ l/well, and male mouse spleen cells suspended in the above medium (2×10^6 /ml) were also added in an amount of 350 μ l/well. Then, trypsinized ST2 cells were suspended (8×10^4 cells/ml) in the above medium containing 4×10^{-8} M vitamin D₃ and 4×10^{-7} M dexamethasone, and the resulting suspension was added in an amount of 350 μ l/well. The plate was incubated at 37°C for 6 days for culture. After the plate was washed with PBS once, the cells were fixed by mixture of 50% ethanol and 50% acetone at room temperature for a minute. After the plate was air-dried, substrate solution was added in an amount of 500 μ l/well in accordance with a protocol of a leukocyte acid phosphatase kit (Sigma Co., Ltd.), and the plate was left to stand at 37°C for 55 minutes so as to cause reaction. By this reaction, cell showing tartaric acid resistant acid phosphatase activity (TRAP activity), which is a specific marker of osteoclasts, were stained. After the plate was washed with distilled water once and air-dried, the number of TRAP positive cells were counted. The results are shown in Table 4. As a result, it was found that both of the rabbit anti-mouse OBM/sOBM polyclonal antibody and H-OBM 9 inhibited derivation of OCL depending on the concentrations of the antibody. It was found that these antibodies had osteoclastogenesis inhibitory activity as in the case of an osteoclastogenesis factor, OCIF/OPG, and were useful as a medicament for treating bone metabolism abnormality.

Table 4

Amount of Antibody Added (ng/ml)	Number of TRAP Positive Multinucleate Cells	
	Rabbit Anti-Mouse OBM/sOBM Polyclonal Antibody	Mouse Anti-Human OBM/sOBM monoclonal antibody (H-OBM 9)
0	1,155 \pm 53	1,050 \pm 45
10	510 \pm 24	650 \pm 25
100	10 \pm 3	15 \pm 4

(average \pm standard deviation, n = 3)

[Example 39]

Osteoclastogenesis Inducing Activity of Trx-OBM

Mononuclear cells were prepared from whole blood sampled from a vein of a normal adult human using Histopaque (Sigma Co., Ltd.) with density gradient technique in accordance with an attached protocol. The mononuclear cells were suspended at a concentration of 1.3×10^6 cells/ml with α -MEM containing 10^{-7} M dexamethasone, 200 ng/ml of macrophage colony stimulating factor (Midori Juji Co., Ltd.), 10% fetal bovine serum and stepwise concentration (0 to 100 ng/ml) of purified Trx-OBM obtained in Example 15. The suspension was added to a 48-well plate in an amount of 300 μ l/well, and the plate was incubated at 37°C for 3 days for culturing cells. Thereafter, the medium was replaced with new (identical with above), and the plate was incubated at 37°C for another 4 days for culturing cells. Cell showing tartaric acid resistant acid phosphatase activity (TRAP activity) were selectively stained by the method described in Example 5, and the number of stained multinuclear cells was counted under the microscope. The results are shown in Fig. 29. As a result, cells showing TRAP activity were hardly detected in the wells containing no Trx-OBM, while TRAP positive multinuclear cells appeared in a manner depend on concentration of Trx-OBM when Trx-OBM was added. Further, these TRAP positive multinuclear cells showed positive result for vitronectin receptor which is a marker of osteoclasts. In addition, when the same culture conditions were used on dentin fragments placed on a 48-well plate, absorption cavities were formed on the surface of dentin fragments only in the presence of Trx-OBM. Thereby, it was revealed that Trx-OBM had activity to induce formation of human osteoclasts.

[Example 40]

Bone Resorption Inhibitory Activity of Anti-OBM/sOBM Antibody

15-day pregnant ddy mice (Nippon SLC Co., Ltd.), 25 μ Ci of [45 Ca]-CaCl₂ solution (Amersham Co., Ltd.) were injected subcutaneously, and fetal bones were labeled with 45 Ca. On the following day, the mice were slaughtered, and their abdomens were opened to remove fetuses from the uteruses. A forelimb was removed from the fetus, the skin and muscle were removed to take out a long bone, and a cartilage on the long bone was also removed so as to leave only the diaphysis of the long bone. Each diaphysis was floated in 0.5 ml of culture medium (BGJb medium (Gibco Co., Ltd.) containing 0.2% bovine serum albumin (Sigma Co., Ltd.)) and cultured at 37°C in the presence of 5%CO₂ for 24 hours. After completion of the pre-culture, the long bone was transferred to a new culture medium (0.5 ml) containing various bone resorption factors (vitamin D₃, prostaglandin E₂, parathyroid hormone, interleukin 1 α) and normal rabbit IgG (100 μ g/ml; as a control) or the rabbit anti-OBM/sOBM polyclonal antibody obtained in Example 28, and then cultured for another 72

hours. After completion of the culture, the long bone was put into 0.5 ml of 5% trichloroacetic acid aqueous solution (Wako Pure Chemical Industries, Ltd.) and treated at room temperature for at least 3 hours so as to be decalcified. To the conditioned medium and the trichloroacetic acid extract (0.5 ml each), 5 ml of scintillator (AQUASOL-2, Packard Co., Ltd.) was added and the radioactivity of ^{45}Ca was measured. The proportion of ^{45}Ca liberated in the culture solution due to bone resorption was calculated. The results are shown in Figs. 30 to 33. As a result, although the vitamin D_3 (10^{-8} M) caused increase of bone resorption activity, the bone resorption caused by the vitamin D_3 was inhibited by addition of the rabbit anti-OBM/sOBM polyclonal antibody in a concentration-dependent manner, and the bone resorption was completely inhibited by addition of the antibody at a concentration of 100 $\mu\text{g/ml}$ (Fig. 30). Further, although bone resorption activity was increased in the presence of prostaglandin E_2 (10^{-6} M) or the parathyroid hormone (100 ng/ml), the bone resorption caused by the prostaglandin E_2 or the parathyroid hormone was almost completely inhibited by the addition of the rabbit anti-OBM/sOBM polyclonal antibody (100 $\mu\text{g/ml}$) (Figs. 31 and 32). Meanwhile, the normal rabbit IgG (100 $\mu\text{g/ml}$) used as a positive control had no effects on the bone resorption by the prostaglandin E_2 and the parathyroid hormone. Further, although bone resorption was induced by the interleukin 1α (10 ng/ml) as well, the bone resorption was inhibited significantly by the rabbit anti-OBM/sOBM polyclonal antibody (100 $\mu\text{g/ml}$) (Fig. 23). From these results, it was revealed that the antibody of the present invention was excellent as a bone resorption inhibitory substance. As a result of conducting the same experiment on H-OBM 9 which was a mouse anti-human OBM/sOBM antibody, it was confirmed that H-OBM 9 had approximately equal bone resorption inhibitory activity to that of the rabbit anti-OBM/sOBM polyclonal antibody.

Industrial Applicability

The present invention provides a novel protein which binds osteoclastogenesis inhibitory factor (OCIF), a method for production thereof, a method for screening a substance which controls expression of the protein by use of the protein, a method for screening a substance which inhibits or modifies an activity of the protein, a method for screening a receptor which binds the protein and transmits an activity thereof, a pharmaceutical composition comprising a substance obtained by said method for screening, an antibody to the protein, and an agent for treating bone metabolism abnormality which is formulated using the antibody.

Furthermore, the present invention provides a DNA which encodes a novel protein (OCIF binding molecule) which binds osteoclastogenesis inhibitory factor (OCIF), a

protein having an amino acid sequence encoded by the DNA, a method for genetically producing a protein which specifically binds to the OCIF by use of the DNA, and an agent for treating bone metabolism comprising the protein. Moreover, methods are provided for screening a substance which controls the expression of the OCIF binding molecule, a method
5 for screening a substance which binds to the OCIF binding molecule and inhibits or modifies an activity thereof, a method for screening a receptor which binds OCIF binding molecule and transmits an activity thereof, and a pharmaceutical composition comprising a substance obtained by said method for screening.

Also provided is: DNA, which encodes a novel human protein (human-derived
10 OCIF binding molecule, human OBM) which binds osteoclastogenesis inhibitory factor (OCIF), a protein and having an amino acid sequence encoded by the DNA, a method for genetically producing a protein which specifically binds OCIF and has a biological activity to support and promote the differentiation and maturation of osteoclasts by use of the DNA, and an agent for treating bone metabolism abnormality comprising the protein.

Also provided are: a method for screening a substance which controls
15 expression of the OCIF binding molecule, a method for screening a substance which binds the OCIF binding molecule and inhibits or modifies an activity thereof, a method for screening a receptor which binds the OCIF binding molecule and transmits the biological activity thereof, and a pharmaceutical composition comprising a substance obtained by said
20 method for screening, as well as an antibody to the human-derived OCIF binding protein, and an agent for preventing and/or treating bone metabolism abnormality which is formulated using the antibody.

Moreover, the present invention provides an antibody (anti-OBM/sOBM
antibody) which recognizes both of the following antigens, i.e., a membrane binding
25 molecule (OCIF binding molecule; OBM) which specifically binds to an OCIF, and a soluble OBM (sOBM) lacking membrane binding sites, a method for production of the antibody, a method for measuring the OBM and sOBM by use of the antibody, and an agent for preventing and/or treating bone metabolism abnormality which comprise the antibody as an active ingredient.

30 The proteins or antibodies presented by the present invention are useful as medicaments, experimental reagents or diagnostic reagents.

Reference to Deposited Microorganisms

(1) Name and Address of Depository Institution:

35 National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry

1-1-3 Higashi, Tsukuba-shi, Ibaragi-ken, Japan (zip: 305)

Date of Deposit:

May 23, 1997

Deposit Number:

5 FERM BP-5953

(2) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of
Industrial Science and Technology of the Ministry of International Trade and Industry

1-1-3 Higashi, Tsukuba-shi, Ibaragi-ken, Japan (zip: 305)

10 Date of Deposit:

August 13, 1997

Deposit Number:

FERM BP-6058

(3) Name and Address of Depository Institution:

15 National Institute of Bioscience and Human-Technology of the Agency of
Industrial Science and Technology of the Ministry of International Trade and Industry

1-1-3 Higashi, Tsukuba-shi, Ibaragi-ken, Japan (zip: 305)

Date of Deposit:

November 5, 1997

20 Deposit Number:

FERM BP-6264

(4) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of
Industrial Science and Technology of the Ministry of International Trade and Industry

25 1-1-3 Higashi, Tsukuba-shi, Ibaragi-ken, Japan (zip: 305)

Date of Deposit:

November 5, 1997

Deposit Number:

FERM BP-6265

30 (5) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of
Industrial Science and Technology of the Ministry of International Trade and Industry

1-1-3 Higashi, Tsukuba-shi, Ibaragi-ken, Japan (zip: 305)

Date of Deposit:

35 November 5, 1997

Deposit Number:

FERM BP-6266

A

DESCRIPTION

NOVEL PROTEIN AND METHOD

AN OCIF-BINDING MOLECULE (OBM), NUCLEIC ACID ENCODING, AND
PROCESS FOR PRODUCING THE PROTEIN

Field of Technology

TECHNICAL FIELD

The present invention relates to a novel protein (OCIF- binding molecule, ~~the protein may be~~; hereinafter called it may be referred to as "OBM"), which binds to osteoclastogenesis inhibitory factor (~~hereinafter it may be called OCIF~~), and a production method to produce this protein thereof. ~~The~~ In addition, the present invention also relates to DNA encoding which this encodes the protein, proteins containing the a protein having an amino acid sequence encoded by this DNA, a method for genetically producing the preparation of this protein utilizing genetic engineering techniques, and a pharmaceutical compositions comprising this protein.

The present invention ~~further also~~ relates to a methods for of screening, using this protein and the DNA, substances to control the for: a substance which controls expression of this protein, substances inhibiting a substance which inhibits or regulating modifies the biological activity of this protein, or receptors transducing a receptor which binds the signal of protein and transmits the activity thereof, methods of using the protein by interacting with this protein, to or the DNA; the substances obtained by the screening these methods; and pharmaceutical compositions comprising the obtained substances. In addition, the present invention also relates to an antibody to the protein, a method for the production thereof, a method for measuring the protein with the antibody, and to pharmaceutical compositions which comprise the resulting substances. an agent comprising the antibody.

The present invention further relates to antibodies against this protein, methods for preparing the antibodies, and pharmaceutical compositions comprising these antibodies.

Background AntiH6, 4400, 839, 5

BACKGROUND ART

Bone metabolism is dependent depends on the overall activity of osteoblasts which responsible control for bone formation, and osteoclasts which control, responsible for bone resorption. Abnormality of It is assumed that bone metabolism abnormality is considered to be caused by an imbalance due to loss of the balance between bone formation and the bone resorption. Osteoporosis As diseases involving bone metabolism abnormality, osteoporosis, hypercalcemia, bone Paget's disease, renal osteodystrophy, chronic rheumatoid arthritis, osteoarthritis, rheumatoid arthritis and the like osteoarthritis are known as diseases accompanying abnormality of bone metabolism. Osteoporosis is a typical disease caused by such abnormality of A representative of these bone metabolism abnormality diseases is osteoporosis. This disease is generated occurs when bone resorption by osteoclasts exceeds bone formation by osteoblasts. The disease and is characterized by a equal decrease in both the bone calcifiedous materials substances and the bone matrix. Although the The mechanism for crisis of this disease is not completely elucidated yet fully clarified, while it is a disease with pain in bone and bone fracture due to the increased fragility of bone. Along with an increase in the population of aged people, this disease causes aches aged people to fracture bone, resulting in bones, makes them fragile, and may result confinement in fracturing bed. This disease is becoming already a social problem because it increases the number of bedridden aged persons as the aged population becomes larger. Development of therapeutic agent, so that medicaments for this treating the disease is are urgently desired needed to be developed. Disease due to a decrease in bone mass It is expected to that osteopenia due to bone metabolism abnormality can be cured treated by suppressing stimulating bone formation, inhibiting bone resorption, accelerating bone formation, or improving the balance between them. That is, bone resorption and formation. Bone formation is expected to be increase stimulated by accelerating proliferation promoting the growth,

differentiation, or activation and functions of osteoblasts, which form are responsible for bone formation, or by suppressing proliferation, the differentiation, or activation of osteoclast precursor cells to osteoclasts which resorb and maturation thereof, or suppressing osteoclast function such as bone-resorbing activity. In recent years At present, strong interest has been directed to hormones, substances of low molecular weight substances, or physiologically active proteins exhibiting having such activities, and energetic basic research and development is underway on these subjects activity are being studied and developed.

Drugs such as As agents for treating bone-relating diseases and shortening treatment periods thereof, a calcitonin-containing agents formulation, the active-form of vitamin D₃-containing agents formulation, hormone agents containing (estradiol, ipriflavone, vitamin K₂) -containing formulation and bisphosphonate compounds have- based compound are already been known as drugs to treat and shorten clinically available. Furthermore, to develop medicaments with less side effects and excellent effectiveness, clinical trials of the treatment period of diseases related to bone. Clinical tests are in progress on active-form of vitamin D₃ derivatives, estradiol derivatives, and bisphosphonate-based compounds of the second and the third generation to develop therapeutic agents with excellent efficacy and minimal side effects have been held.

However, therapies since such methods for treatment using these agents were found drugs are not necessarily satisfactory sufficient in terms of efficacy effectiveness and therapeutic results. Development of treatment, novel therapeutic agents which medicaments that are safer and with have higher efficacy is urgently desired effectiveness have been expected to be developed. Some agents Moreover, among medicaments used for the in treatment of diseases related to bone metabolism diseases, there are those which can be used only limitedly for treating a restricted kind of disease due to their side effects thereof. Furthermore In addition, treatments using two or more agents in combination are currently the mainstream in the treatment of diseases related at present, to treat bone metabolism diseases such as osteoporosis, treatment with combined use of more than one medicament is currently usual. From such a point of view, development of drugs a medicament having different action mechanisms different from

those of ~~the conventional drugs, and exhibiting a ones~~ with higher efficacy/effectiveness and ~~minimal/less~~ side effects ~~is desired~~ have been expected to be developed.

As ~~mentioned/described~~ above, the cells ~~controlling/responsible~~ for bone metabolism are osteoblasts and osteoclasts. These cells ~~are~~ It is known to have close mutual interactions called "that these cells closely interact with each other, and this phenomenon is regarded as coupling". Specifically ~~That is~~, it has been reported that the differentiation and maturation of osteoclasts are stimulated or suppressed by cytokines such as Interleukins, interleukins 1 (IL-1), 3 (IL-3), 6 (IL-6), and 11 (IL-11), granulocyte-macrophage-colony-stimulating factors (GM-CSF), macrophage-colony-stimulating factors (MGM-CSF), Interferon- γ gammas (IFN- γ), tumor necrosis factors α (TNF- α), and transforming growth factor- β factors β (TGF- β), and the like, which are secreted by osteoblastic stroma from osteoblast-like stroma cells ~~are known to accelerate or suppress differentiation or maturation of osteoclasts~~ (Raisz: Disorders of Bone and Mineral Metabolism, 287- to 311, 1992; Suda *et al.*: Principles of Bone Biology, 87- to 102, 1996; Suda *et al.*: Endocrine Reviews, 4, 266- to 270, 1995, 1955, Lacey *et al.*: Endocrinology, 186, 2369- to 2376, 1995). It ~~has been reported~~ is known that osteoblastic/osteoblast-like stromal cells play an important role in the differentiation and maturation of osteoclasts, as well as in and expression of mature osteoclast functions/function, such as bone resorption ~~by mature osteoclasts~~, through cell-intercellular binding to cell contact with immature osteoclast precursors/precursor cells of osteoclasts or (mature) osteoclasts.—A factor called As a factor involved in osteoclastogenesis by the intercellular binding, a molecule known as osteoclast differentiation factor (ODF), (Suda *et al.*: Endocrine Rev. 13:13, 66- to 80, 1992; Suda *et al.*: Bone 17, 87S- to 91S, 1995) which is thought to be expressed on the membrane of osteoblastic/the osteoblast-like stromal cells and involved in the formation of osteoclasts through cell-to-cell contact cell is predicted. According to this hypothesis/assumption, an ODF α receptor is ~~present~~ for ODF exists in the osteoclast precursor cells of osteoclasts/cell. However, ~~so far neither the ODF nor~~ and this receptor ~~has been~~ are not yet either purified or identified.—There, and there are also no reports relating to/on their characteristics, action mechanism/mechanisms or structure/end structures. Thus As just described, the mechanism ~~involved in~~ for differentiation and maturation of osteoclasts has

not yet been sufficiently elucidated. Clarification fully understood yet, and it is expected
that full understanding of this mechanism will greatly significantly contribute not only
to the basic medicine, field of experimental medicines but also to the
development/developments of novel drugs/agents for the treatment of diseases associated
with treating bone metabolism abnormality of bone metabolism, based on the novel action
mechanism.

The Under the circumstances, the present inventors have conducted/made
extensive/intensive studies in view of this situation and discovered an/found
osteoclastogenesis inhibitory factors (OCIF) in at the culture broth/solution of human
embryonic/fetal lung fibroblast, fibroblasts IMR-90 (ATCC Deposition No. CCL186)
(WO 96/26217).

The Then, the present inventors have been successful/succeeded in DNA cloning
DNA encoding of OCIF, production of a recombinant OCIF in using an animal cells, and
confirmation of in vivo pharmaceutical/medicinal effects/virtues (bone metabolism
improving effect on bone metabolism, etc.) of the recombinant OCIF. OCIF is expected
to be used as an agent for a medicament that has higher effectiveness and causes less side
effects than the prevention or treatment of conventional one and can prevent and treat
diseases related to abnormality of associated with bone metabolism, with higher efficacy
than conventional drugs and less side effects abnormality.

Disclosure of the Invention**DISCLOSURE OF THE INVENTION**

The present inventors have intensively searched for the existence of a protein
which/that binds to osteoclastogenesis inhibitory factor (OCIF) and discovered by using
OCIF. As a result, the inventors have found that an OCIF- binding protein is specifically
expressed on the an osteoblastic/osteoblast-like stromal cells cultured in the presence of a
bone resorption factors such as the active-form of vitamin D₃ and parathyroid hormone
(PTH). In addition/Furthermore, the present inventors have investigated as a result of
studying the characteristics and physiological functions of this OCIF- binding protein and
found that, the protein exhibits/was found to have biological activity of as a factor which
supports or promotes the so-called osteoclast differentiation and maturation from factor,
associated with differentiation of immature precursors of osteoclast precursor cells to
osteoclasts. These findings have led to the completion of the present invention and

maturation thereof. ~~Further investigation into the protein of the~~ The present invention has
proven been completed based on this finding. Moreover, as a result of further studying
the protein of the present invention, the present inventors have found that this the novel
membrane protein is an important protein controlling which leads the differentiation and
maturation of osteoclasts from immature precursors of osteoclast precursor cells to
osteoclasts by osteoblast-like stromal cells in a co-culture system of the
osteoblastic osteoblast-like stromal cells and spleen cells. The ~~success in~~ successful
identification and, isolation and purification of the protein which functions as a factor
supporting or promoting which supports and promotes the differentiation and maturation
of osteoclasts in the present invention has enabled a screening for of a novel
medicine useful for abnormality of agent for treating bone metabolism abnormality, based
on a mechanism of for bone metabolism utilizing in a living subject, using the protein of
the present invention.

~~Accordingly~~ Therefore, an object of the present invention is to provide a novel
protein (OCIF- binding molecule or; OBM), which binds to osteoclastogenesis inhibitory
factor (OCIF), and a method to ~~produce this protein for the production thereof.~~ Another
object of the present invention is to provide DNA ~~encoding this which encodes the~~
protein, ~~proteins containing a protein having an amino acid sequence encoded by this~~
DNA, a method for genetically producing this protein-utilizing genetic engineering
techniques, and a pharmaceutical compositions comprising this protein. Furthermore,
further another object of the present invention is to provide ~~methods an agent for~~
preventing and/or treating bone metabolism abnormality comprising the protein.
Moreover, another object of the present invention is to provide: a method of screening
substances for: a substance which controls expression of this protein using this the protein
and the DNA, substances inhibiting a substance which inhibits or regulating modifies the
biological activity of this protein, receptors transducing or a receptor which binds the
action protein and transmits the activity of the protein by binding to; a method of using
the protein, substances and DNA thereof; a substance obtained by the at
screening method; and pharmaceutical compositions which comprises these comprising
the obtained substances. ~~A still further~~ Furthermore, another object of the present
invention is to provide ~~antibodies against this protein, methods for preparing an antibody~~

to the antibodiesprotein, a method for production thereof, a method for measuring the protein using the antibody, and a medicament (agent; pharmaceutical compositionscomposition) comprising these antibodiesy.

The protein of the present invention ~~has~~shows the following physicochemical properties and biological activity. That is, (a) Affinity: the protein specifically binds to the osteoclastogenesis inhibitory factor (OCIF) and exhibits~~has~~ high affinity to OCIF (a dissociation constant, a Kd value, on a cell membrane: Kd=surface, is not larger than 10⁻⁹ M or less) ; (b) Molecular weight: has the protein shows a molecular weight of approximately~~about~~ 30,000- to 40,000 when as determined~~measured~~ by SDS-polyacrylamide gel-electrophoresis (SDS-PAGE) under non-reducing conditions, and shows an apparent molecular weight of approximately~~about~~ 90,000- to 110,000 when cross-linked~~crosslinked~~ to with a monomer-form-type OCIF; and (c) Biological the protein has an activity: exhibits activity supporting or promoting osteoclast to support and promote the differentiation and maturation of osteoclasts in a co-culture system of the mouse osteoblastic~~osteoblast-like~~ stromal cells and mouse spleen cells in the presence of bone resorption factors such as the active-form of vitamin D₃ and parathyroid hormones (PTH).

~~As a representative~~ in vitro culture system for osteoclastogenesis, a co-culture system of ST2, a mouse osteoblastic-derived osteoblast-like stromal cell line, ST2, and mouse spleen cells in the presence of the active-form of vitamin D₃ or PTH is well known as a typical in vitro culture system for osteoclast formation. The cells expressing that express the protein of the present invention can be determined~~obtained~~ by testing~~examining~~ the binding ability of OCIF to a mouse osteoblastic~~osteoblast-like~~ stromal cells or mouse spleen cells cultured in the presence or absence of the active-form of vitamin D₃₋₃ to OCIF. The protein of the present invention is specified~~identified~~ as the~~a~~ protein which is induced specifically induced on the~~an~~ osteoblastic~~osteoblast-like~~ stromal cells cultured in the presence of an osteotropic bone resorption factors such as the active-form of vitamin D₃ or PTH. In addition~~Further~~ , the~~in~~ protein~~consideration~~ of the invention can be specified as a protein exhibiting biological activity supporting or promoting differentiation and maturation of osteoclasts from the following results. That is, the facts that osteoclast formation is inhibited dose dependently by the addition of 1 to

~~40 ng/ml of OCIF to the above-mentioned co-culture system in the presence of the active-form of vitamin D₃, the time-course of~~ in a dose-dependent manner within a range of 1 to 40 ng/ml of OCIF; that there is an intimate correlation between change in expression of the protein of the present invention protein induced on the ST2 cells in the presence of the active-form of vitamin D₃ well-correlates and the change in osteoclast formation with the the passage of time-course of osteoclast formation in the co-culture. In addition,; that the amount of protein of the present invention protein expressed on a ST2 cells correlatesponds with to the capabilityintensity of thean cellsability to support the osteoclast formation,; and thethat osteoclast formation is completely inhibited by binding of OCIF(s) to the protein of the present invention protein on the ST2 cells-completely suppresses, the protein of the present invention is identified as a protein having biological activity (effect) to support and promote the differentiation and maturation of osteoclasts formation.

The affinity of the protein of the present invention ~~to~~ for OCIF can be ~~evaluated~~ assessed by labeling OCIF and ~~examining~~ testing the binding activity of the labeled OCIF to the surface of an animal cell membrane. OCIF can be labeled by a ~~conventional~~ commonly used protein-labeling method such as labeling with a radioisotope or fluorescence labeling. Labeling of tyrosine residues with ¹²⁵I can be given as a specific For instance, an example of labeling of the-OCIF with a radioisotope. Labeling is ¹²⁵I labeling at a tyrosine residue, and labeling methods such as i odogen method, chloramine T method, and enzymatic and enzyme method can be utilized. can be employed thereto. The binding ~~of the~~ activity of the thus labeled OCIF to the surface ~~membrane of~~ of an animal cell membrane can be examined in accordance with a commonly used method, and the amount of nonspecific binding can be testedmeasured by a conventional method. The additionadding 100 to 400 times excess amounts of unlabeled OCIF to the medium used for the binding assay to a concentration, 100 to 400 times the concentration of labeled OCIF, ensures measurement of non-specific bindingexperiment. The amount of specific binding of OCIF ~~can be~~ is calculated by subtracting ~~the amountthat~~ of non-specificthe nonspecific binding from ~~thethat~~ of total amount of binding of the labeled OCIF. The affinity ~~of the protein~~ (for OCIF) of the present ~~invention~~ protein expressed on ~~thea~~ a cell membrane ~~to OCIF can be evaluatedis~~

assessed by conducting the amount test with various amounts of the labeled OCIF and analyzing the amount of the specific binding by Scatchard plot. The determined affinity of the protein of the present invention for OCIF is approximately about 100- to 500 pM. Thus, the protein of the present invention is specified/identified by a protein having such high affinity (the dissociation constant on cell membrane, the K_d value, on a cell membrane is not larger than 10^{-9} M or less) to osteoclastogenesis inhibitory factor (for OCIF). The molecular weight of OBM can be accessed is measured by use of gel filtration chromatography, SDS-PAGE, or the like. To measure the molecular weight more accurately, SDS-PAGE is preferred in order to accurately determine the molecular weight. The preferably used, and OBM is specified/identified as a protein having a molecular weight of approximately about 40,000 ($40,000 \pm 4,000$) under reducing conditions.

The protein of the present invention can be obtained from a mouse osteoblastic/osteoblast-like stromal cell line, ST2, a mouse preadipocyte fat cell line, strain PA6, or human osteoblastic/osteoblast-like cell lines, or other concentrated osteoblastic/osteoblast-like cells selected/obtained from mammals such as humans, mice, or rats/mouse and rat. As the And, substances that are required to induce expression of/express the protein of the present invention, osteotropic on these cells may be bone resorption factors such as the active-form of vitamin D_3 (calcitriol), parathyroid hormone (PTH), interleukins (IL)-1, IL-6, IL-11, Oncostatin M, and leukemia inhibitory cell growth inhibiting factor (LIF)-can be given. These As for the amounts of these substances can be added in, it is desirable to use the concentration of 10^{-8} M (active-form of vitamin D_3 and/or PTH), in an amount of 10^{-8} M; the IL-11 and the oncostatin M in amounts of 10 ng/ml (IL-11), or and 1 ng/ml (Oncostatin M), respectively; and the IL-6 is preferably used at a concentration in an amount of 20 ng/ml with 500 ng/ml of IL-6 soluble IL-6 receptor. Preferably, confluent It is preferable to use cells of/obtained by culturing mouse osteoblastic/osteoblast-like stromal cell line, ST2, cultured in α -MEM medium to which containing 10^{-8} M of the active-form of vitamin D_3 , 10^{-7} M of dexamethasone, and 10% fetal bovine fetal serum were added can be used for at least one week until the cells become confluent. Thus cultured cells may be removed and collected by scraping

with using a cell scraper or the like. Moreover, the collected cells may be stored at -80°C until use.

The protein of the present invention can be purified efficiently from the membrane fractions of the thus collected cells. The membrane fractions can be prepared by in accordance with a conventional common method which is used to prepare intracellular organelle. Various types used for fractionation of protease inhibitors may be added to the organelles. As a buffer solution used for the used in preparation of the membrane fractions fraction, various protease inhibitors may be preferably added. Examples Illustrative examples of the protease inhibitors to be added include serine protease inhibitors, thiol protease inhibitors, and metalloprotease inhibitors, such as PMSF, APMSF, EDTA, o-phenanthroline, leupeptine, pepstatin A, aprotinin, and a soybean trypsin inhibitor are given as specific examples. A Dounce homogenizer To crush the cells, a polytron Dounce homogenizer, a polythoron homogenizer, an ultrasonicator or a ultrasonic processor the like can be used to homogenize the cells. The cell homogenate is crushed cells can be suspended in a buffer solution containing 0.5 M of sucrose and centrifuged at 600 X g for 10 minutes at 600 x g, so as to separate the cell nuclei and undischarged cells as precipitate a precipitated fraction. The supernatant is centrifuged After further centrifugation at 150,000 X g for 90 minutes at 150,000 x g to obtain, a membrane fractions fraction can be obtained as precipitate a precipitated fraction. The By treating the thus obtained membrane fraction is treated by with various types of detergents to efficiently solubilize and extract surfactants, the protein of the present invention from existing on the cell membrane can be solubilized and extracted, efficiently. Detergents For solubilization, various surfactants which are commonly conventionally used to solubilize in solubilization of cell membrane proteins, such as CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), Triton X-100, Nikkol, and n-octyl glycoside octylglycoside, can be used. Preferably, The protein of the present invention is preferably solubilized by adding 0.5% CHAPS is added to the membrane fraction protein and agitating the mixture is at stirred 4°C for 2 hours at 4°C to solubilize the protein of the present invention. The sample By centrifuging the thus prepared is centrifuged sample at 150,000 x X g for 60 minutes to obtain, the solubilized membrane fraction can be obtained as a supernatant.

The protein of the present invention can be purified efficiently from the thus obtained solubilized membrane fraction with a, using an OCIF-immobilized column, gel, or resin coupled with OCIF. The As immobilized the OCIF may to be used in the immobilization, that isolated from at the culture broth solution of human embryonic fetal lung fibroblasts, IMR-90, in accordance with a method described in WO 96/26217 or rOCIF prepared using that obtained by genetic engineering techniques (rOCIF) can be used. This rOCIF can be prepared obtained by introducing incorporating the corresponding human cDNA, rat or mouse cDNA, or rat cDNA into an expression vector according to in accordance with a conventional common method, transducing expressing the constructed vector in OCIF with animal or insect cells such as CHO cells, BHK cells, or and Namalwa cells, or in insect cells to produce rOCIF, and then purifying rOCIF it. Obtained The thus obtained OCIF has shows a molecular weight of approximately about 60 kDa (monomer-form type) or and a molecular weight of about 120 kDa (dimer-form type). The A dimer-form type OCIF is preferable for preferably used in the immobilization. Given as examples of the gels and resins to which As a gel or a resin for immobilizing OCIF is immobilized are, ECH Sepharose SEPHAROSE® 4B, EAH Sepharose SEPHAROSE® 4B, Tthiopropyl Sepharose SEPHAROSE® 6B, CNBr-activated Sepharose SEPHAROSE® 4B, activated CH Sepharose SEPHAROSE® 4B, Epoxy activated Sepharose SEPHAROSE® 6B, activated thiol Sepharose SEPHAROSE® 4B (these are manufactured by products of Pharmacia Co., Ltd.), TSK Kgel AF-Epoxy Toyopal TOYOPAL 650, TSK Kgel AF-Amino Toyopal TOYOPAL 650, TSK Kgel AF-Formyl Toyopal TOYOPAL 650, TSK Kgel AF-Carboxy Toyopal TOYOPAL 650, TSK Kgel AF-Tresyl Toyopal TOYOPAL 650 (these are manufactured by Tosoh Corporation products of Toso Co., Ltd.), Amino-Cellulofine, CELLULOFINE™ Ccarboxy-Cellulofine CELLULOFINE™, FMP activated Cellulofine CELLULOFINE™, Formyl-Cellulofine formyl-CELLULOFINE™ (these are manufactured by Seikagaku Kogyo products of Sei Kagaku Kogyo Co., Ltd.), Affigel AFFIGEL 10, Affigel AFFIGEL 15, 15 and Affiprep AFFIPREP 10 (these products of Bio-Rad Co., Ltd.) are manufactured by BioRad Co.) available. As columns to which Furthermore, as a column for immobilizing OCIF is immobilized, HiTrap HITRAP® NHS-activated column (Pharmacia Co., Ltd.), TSKgel Tresyl-5PW (Tosoh

~~Corporation Toso Co., Ltd.), etc. or the like can be given used.~~ As a specific example of the method for immobilizing OCIF to a HiTrap with the HITRAP® NHS-activated column (1 ml, Pharmacia Co., Ltd.), the following method ~~can be given is presented.~~ Specifically That is, 1 ml of 0.2 M NaHCO₃/0.5 M NaCl solution (pH 8.3) solution containing 13.0 mg of OCIF is injected applied to the column and allowed to ~~perform~~ undergo a coupling reaction at room temperature for 30 minutes. Then, after 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH 4.0) are sequentially applied to the column. Then, the column is again washed with respectively, 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) is applied again, and then the column is allowed left to stand for one hour at room temperature for 1 hour so as to block inactivate excess active groups. The Thereafter, the column is sequentially washed twice with 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH 4.0), and then washed replaced with 50 mM Tris/1 M NaCl/0-10.1% CHAPS solution buffer (pH 7.5); thereby obtaining a. Finally, an OCIF-immobilized column can be prepared. Using the prepared OCIF-immobilized column. The, gel or resin, the protein of the present invention can be efficiently purified by a OCIF-immobilized column prepared in this manner, or an OCIF-immobilized gel or resin efficiently. It is desirable to add To prevent the proteolysis of the protein of the present invention, the above various above-mentioned protease inhibitors may also be added to the buffer solutions used for their purification of the protein to suppress degradation of the protein of the present invention. The protein of the present invention can be purified by loading After applying the above-mentioned solubilized membrane fraction onto the an OCIF-immobilized column or by mixing the solubilized membrane fraction with the an OCIF-immobilized gel or resin; and eluting subsequently stirring the mixture so as to cause the fraction to be adsorbed, the protein of the present invention can be eluted from the OCIF-immobilized column, gel, or resin with using an acid, various protein -denaturing agents, a cacodylate buffer; and or the like. It is desirable to use an acid for elution and to neutralize immediately after elution to To minimize denaturation of the protein of the present invention, it is preferable to neutralize the eluate immediately using a base. As the acid, an acid buffer solution used for elution, 0.1 M glycine-hydrochloric acid buffer solution (pH 3.0), 0.1 M glycine-

hydrochloric acid buffer solution (pH 2.0), and 0.1 M sodium citrate buffer solution (pH 2.0), and the like can be given used, for example.

The purified protein of the present invention can be further purified by conventional use of a method which is conventionally employed in purification methods used for purification of various of proteins from biological materials and by samples, through various purification methods utilizing operations taking advantages of the physicochemical properties of these protein of the present invention. To concentrate solutions containing a solution of the protein of the present invention, conventional techniques a method which is conventionally used in the protein purification process for proteins such as ultra-filtration, e.g., ultrafiltration, freeze -drying, and salting-out, can be used. Ultra-filtration Preferably, ultrafiltration based on centrifugation with Centricon-CENTRICON®-10 (BioRadAmicon Co.), for example, and the like is preferably used. As Furthermore, as a means for the purification of purification, various techniques methods conventionally utilized for the used in protein purification of proteins, such as using ion exchange chromatography, gel filtration chromatography, hydrophobic chromatography, reversed phase chromatography, and preparative electrophoresis, are and the like can be used in combination. More specifically, it is possible to purify the protein of the present invention can be purified by a combined combination use of gel filtration chromatography with Superose-SUPEROSE®-12 column (Pharmacia Co., Ltd.) and the like and reverse phase chromatography. To detect Moreover, the protein of the present invention in during the purification process, the binding can be detected by analyzing activity of the protein of the present invention to bind the immobilized OCIF is examined or the material bound to the immobilized OCIF is by immuno precipitated precipitation of OCIF-binding substances with an anti-OCIF antibody and analyzed followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

The thus obtained protein of the present invention is useful, due to its activity, as an agent medicaments, e.g., as agents for treating diseases caused by abnormality of bone metabolism abnormality such as osteopetrosis, or as a reagent for research experimental and diagnosis of these diseases diagnostic reagents.

The Furthermore, the present invention further relates provide to DNA encoding which encodes a novel protein (OCIF- binding molecule or, OBM) which binds

to osteoclastogenesis inhibitory factor (OCIF), ~~proteins containing the~~ protein having an amino acid sequence encoded by this DNA, a method for the preparation of this protein genetically producing a protein which specifically binds OCIF by use of the genetic engineering technique ~~protein, and pharmaceutical compositions~~ an agent for treating bone metabolism abnormality comprising this protein. Furthermore In addition, the present invention ~~provides methods~~ relates to a method for screening ~~substances to regulate a substance which controls~~ expression of OBM, a method for screening substances inhibiting or modifying the biological activity of a substance which binds OBM and inhibits or modifies an effect thereof, ~~or a method for screening receptors transducing the action of a receptor which binds OBM by binding to OBM and transmits an effect thereof,~~ and pharmaceutical compositions ~~which comprises~~ comprising a substances obtained as a result of these methods for screening.

The novel protein OBM ~~which is encoded by the DNA of the present invention~~ has shows the following physicochemical properties and biological activity. That is, (a) binds the protein specifically ~~to binds~~ osteoclastogenesis inhibitory factor (OCIF), (b) has the protein shows a molecular weight of approximately about 40,000 (\pm 4,000) when as determined measured by SDS-PAGE under reducing conditions, and shows an apparent molecular weight of approximately about 90,000- to 110,000 when crosslinked ~~to with a monomer-form type~~ OCIF; and (c) ~~exhibits the protein has an~~ activity supporting or promoting to support and promote differentiation and maturation of osteoclasts.

Human osteoclastogenesis inhibitory factor (OCIF) ~~which is used as a probe to identify for assessing the properties of OBM in identification of the DNA encoding OBM,~~ the OCIF- binding molecule OBM of the present invention, and ~~to evaluate properties of OBM can be isolated from at the culture broth solution of a human embryonic fetal lung fibroblast cell line fibroblasts, IMR-90, according to in accordance with WO No. 96/26217. Recombinant~~ For isolation and identification of the DNA encoding OBM, recombinant human OCIF, recombinant mouse OCIF, recombinant rat OCIF, and the like can also be used for the isolation and identification of the DNA coding OBM. These recombinant OCIF proteins can be produced obtained by inserting incorporating the corresponding DNA fragments encoding these proteins into an expression vectors according to conventional methods vector in accordance with a commonly used method,

subsequently expressing in OCIF with animal or insect cells such as CHO cells, BHK cells, or and Namalwa cells, or in insect cells, and then purifying them it.

As a method Methods for isolating cloning a cDNA encoding a which encodes the target protein (cDNA cloning) include, the a method comprising determination the steps of determining a partial amino acid sequence of the protein and isolation of isolating the target cDNA by hybridization utilizing the based on a nucleotide sequence corresponding to the amino acid sequence can be employed. Another available method, even in comprises the ease where steps of constructing a cDNA library with an expression vector, regardless of whether or not the amino acid sequence of the protein is not known, comprises constructing a cDNA library in a expression vector, subsequently introducing the cDNA it into cells, and then screening for the presence and absence of expression of the target protein to and isolating the objective desired cDNA (expression cloning method, D' Andrea *et al.*: Cell 57, 277- to 285, 1989; Fukunaga *et al.*: Cell 61, 341- to 350, 1990) (expression cloning method). In the expression cloning method, suitable host cells such as bacteria bacterial, yeast, animal cells, and the like are selected depending on the objective. In many cases, animal cells are selected and used as the host cells for according to the purpose. For cloning a cDNA encoding a which encodes a protein such as the protein of the present invention which is considered to be present in on the surface of animal cell membrane surface. Normally as in the present invention, host animal cells showing are often used as hosts. Furthermore, hosts with high efficiency for introducing DNA transfection and achieving expression of expressing the introduced DNA at high levels are selected conventionally used. One of such animal the cells having such characteristics is the a monkey kidney cell cell line, COS-7, used in the present invention. Because Since SV40 large T antigen is expressed in the COS-7 cells, plasmids having a plasmid which has a replicator of SV40 can be origin of replication are present in the cell as an multicopy episome of multiple copies in the cell, so that a high level of where by higher expression is than usual can be expected. In addition Moreover, because expression of a target protein by COS-7 cells reaches as since the maximum expression level is reached within a few days after the introduction of DNA, the cell is COS-7 cells are suitable for rapid quick screening. A In combination of this host cell with a plasmid capable suitable or for high expression ensures gene expression of, this host cell enables an

extremely high level of gene expression. The promoter is a factor exhibiting the greatest influence on the expression of a gene on a plasmid is which has the most significant effects on the amount of gene expression. As a promoter, ~~Promoters such as SR α~~ suitable for high level of expression, SR α promoter and cytomegalovirus-derived promoters are often used as high expression promoters. ~~To Screening screen methods for cloning the cDNA encoding of a the membrane protein by the include expression cloning strategy, screening procedures such as binding method, panning method; or and film emulsion method are used.~~

The present invention relates to DNA encoding, which encodes the protein (OBM) which specifically binds to OCIF, isolated (OBM), obtained by the a combination of the expression cloning strategy method and the screening by the binding method, to the protein expressed protein therewith, and to a screening of physiologically a biologically active substances using with the DNA or the expressed protein. OBM encoded by the DNA of the present invention can be detected by labeling OCIF and testing subsequently examining the binding activity of the labeled OCIF to membrane the surface of an animal cell membrane. OCIF can be labeled by a conventional method for labeling method protein such as labeling with a radioisotope-labeling method or fluorescentce labeling method which. An example of labeling OCIF with radioisotope is used for ¹²⁵I labeling ~~common proteins~~. Labeling at tyrosine residues by ¹²⁵I can be given as a, and specific example of labeling OCIF with a radioisotope. Labeling methods such as the ~~iodogene include~~ Iodogen method, chloramine T method, and enzymatic method can be utilized. The binding activity of thus labeled OCIF to the surface of an animal cell membrane surface can be tested assessed in accordance with a commonly used method. Furthermore, an amount of nonspecific binding can be measured by conventional methods. The addition adding 100 to 400 times excess amount of unlabeled OCIF to the medium used for the test to a concentration, 100 to 400 times the concentration of labeled OCIF, enables quantification of the amount of non-specific binding experiment. The amount of specific binding of OCIF ~~can be~~ is calculated by subtracting that of the amount of non-specific nonspecific binding from that of the total amount of binding of the labeled OCIF.

~~The present inventors~~ Based on an assumption that there is interaction between thea factor, which is involved in differentiation of osteoclasts and OCIF. Based on this assumption, to isolate the protein to which recombinant interacts with OCIF binds, the inventors have screened thean expression library prepared from the mRNA of a mouse osteoblastic osteoblast-like stromal cell line, ST2, aeording to with recombinant OCIF in accordance with the following method in order to separate the protein which binds OCIF. Specifically, DNA synthesized using from the mRNA of ST2 mRNA cells was inserted into an expression vector for an animal cell cell, and the vector with the insert was introduced they were transduced (transfected) into COS-7 monkey kidney COS-7 cells. The objective Using ¹²⁵I-labeled OCIF as a probe, the target protein expressed on the COS-7 cells was screened using OCIF labeled with ¹²⁵I as a probe. As a result, DNA encoding which encodes the protein which binds that specifically to binds OCIF was isolated. The could be separated, and then the nucleotide sequence of the DNA encoding which encodes this OCIF- binding molecule (OCIF- binding molecule; OBM) was then determined. Moreover Furthermore, it has been found that OBM encoded by thise DNA was found to bind strongly and specifically and strongly to binds OCIF, on the cell membrane.

Comparatively An example of DNA hybridization under relatively mild conditions for hybridization of DNA in the present invention are the conditions, for example, wherein is that after DNA is transferred to a nylon membrane and immobilized thereto according to conventional methods and fixed in accordance with a common method, it is hybridized in a buffer solution for with a radio-labeled DNA as a probe in a hybridization with a probe DNA labeled with an isotope buffer at a temperature of 40- to 70°C for about 2 hours to overnight, followed by and then washed in with 0.5 ~~X~~ SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 45°C for 10 minutes. Specifically More specifically, Highbond after DNA is transferred and fixed to a nylon membrane, HYBOND® N (Amersham Co.) is used as the nylon membrane to transfer and immobilize DNA thereon, Ltd. (DNA), in accordance with a conventional method, it is then hybridized with a probe DNA ³²P-labeled with ³²P DNA as a probe in a rapid hybridization buffer Rapid Hybridization Buffer (Amersham Co., Ltd.) at 65°C for 2

hours, followed by and then washed with 0.5 \times SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 45°C for 10 minutes.

~~As a representative *in vitro* culture system for osteoclastogenesis, a co-culture system of mouse-derived osteoblastic osteoblast-like stromal cell line, ST2, and mouse spleen cells in the presence of the active-form of vitamin D₃ or PTH is well known as a typical *in vitro* culture system for osteoclast formation. The protein OBM of the present invention is specified as the identified as a protein which is induced specifically induced on the osteoblastic stromal cells an osteoblast-like stroma cell cultured in the presence of an agent which accelerates bone resorption factors such as an active-form of vitamin D₃ or PTH. In addition~~ Furthermore, because of the fact that formation of since osteoclasts formation is stimulated by the addition of adding the protein encoded by the DNA of the present invention to a culture system of mouse spleen cells cultured even in the absence of the active-form of vitamin D₃ or PTH, OBM which is encoded by the DNA of the present invention is considered to be involved in the differentiation and maturation of osteoclasts.

~~Recombinant~~ A recombinant OBM can be produced by inserting the DNA of the present invention into an expression vector so as to construct prepare a plasmid for expressing OBM, and then introducing and expressing the plasmid into various cells or microorganisms to express recombinant OBM and microbial strains. As a host in which recombinant OBM is expressed, mammalian cells such as COS-7, CHO, Namalwa, or bacteria such and the like can be used as mammalian hosts cells for expression, and *Escherichia coli* (*E. coli*) and the like can be used as bacterial host cells for expression. In such a case, the recombinant OBM may can be expressed as a membrane-bound-form protein using the full length of DNA or as a secretionry-form type or as a solubilized-type (soluble-form type) protein by removing a part of the portion DNA encoding the a transmembrane membrane-binding domain from the full length. The thus produced recombinant OBM can be purified efficiently purified using a suitable in combination of with conventional methods used in protein purification methods used for common proteins, such as affinity chromatography using an OCIF-immobilized columns, ion exchange chromatography, and gel filtration chromatography and the like. The thus obtained protein of the present invention is useful, due to its activity, as an

agent medicaments, e.g., as agents for treating diseases caused by abnormality of bone metabolism abnormality such as osteopetrosis or as a reagent for research and diagnosis of such diseases as experimental or diagnostic reagents.

The following screening operations can be carried out using the protein OBM encoded by the DNA of the present invention enables: (1) screening of substance a substance which regulate controls expression of OBM₂; (2) screening of substance a substance which specifically bind to binds OBM and inhibit inhibits or modifies the biological activity of OBM₂; and (3) screening of protein a protein (OBM receptor) which are present in osteoclast exists on a precursor cell cell of osteoclasts and transduce emits the biological activity of OBM₂ and (OBM receptor₄). It is also possible to develop as well as developments of antagonists and agonists using this OBM receptor. In the combinatorial chemistry using the above-mentioned OBM or OBM receptor, a peptide library used for the screening of the antagonists required to identify an antagonist or agonists can be prepared by in accordance with the following method. Specifically, one of the specific methods. One of them is a split method (Lam *et al.*; Nature 354, 82- to 84, 1991). According to In this method, synthetic carriers (beads) each comprising a specific amino acid (unit) are bound thereto are prepared to amino acids (units), separately for all units. The synthesized carriers Then, these synthetic beads are mixed altogether together and divided into portions an equal to the number of the units. Then, the next units are and then bound to the subsequent units. This procedure is repeated “By repeating this operation n” times to produce, a library containing carriers to in which “n” units are bound to the carriers is prepared. According to this synthetic method, each carrier pool has one type of Such an operation allows the synthesis of only one sequence per one group of the carriers. Therefore Hence, it is possible to identify a peptide specifically binding to the protein of the present invention by selecting the pool which gives a signal when a positive carrier group is selected in this said method for screening method using by use of the protein of the present invention; and determining then the amino acid sequence of the thereof is determined, a specifically binding peptide bound on the pool can be identified. Another As another method, is a phage display method which utilizes phage carrying can be used. In this method, synthetic DNA which encode genes encoding random peptides with random amino acid sequences are expressed using phage.

~~The~~ While this method has ~~the~~ an advantage of ~~increasing the~~ that it can archive a larger number of molecules in ~~the~~ a library as ~~compared with~~ than the above-mentioned synthetic peptide-library method, ~~but it also has the~~ a disadvantage that the kind of less variety for a given number of peptides per molecules is not as varied because there can be particular peptides having sequences which are missing that phages don't prefer do not exist in the library if the phages are unable to express those sequences. In the phage display method, as in the case of the split method, using a screening system using ~~with~~ the protein of the present invention ~~can also be applied to determine the nucleotide sequence encoding the peptide.~~ That is, the phage specifically binding to the protein of the present invention ~~is~~ there to are concentrated by panning, ~~the selected.~~ Thus obtained phage ~~is~~ are amplified in ~~E. coli~~ E. coli, and further, the nucleotide sequence encoding the peptide is determined. ~~In addition~~ Furthermore, when it is desired that a specific peptide exhibiting high specificity and having high affinity to ~~for~~ OBM or OBM receptor ~~can be~~ is screened from a peptide library using the screening ~~systems mentioned~~ system of the above in (2) ~~and~~ or (3), a specific peptide having a very high affinity can be obtained by screening a positive carrier or phage in the co-presence of OBM or OCIF while increasing the ~~or~~ OBM with a change of concentration of OBM or OCIF. Only positive carrier pools or phages are selected in this manner. For example, screening of a peptide agonist of low molecular weight peptide agonists exhibiting ~~having~~ an EPO (erythropoietin)-like activity ~~were screened from a~~ varied peptide library using a receptor ~~of~~ with an erythropoietin (EPO) which is a hematopoietic hormone) receptor, the tertiary analysis of a three-dimensional structure thereof, and the production of this a substance ~~was analyzed, and based on this tertiary structure,~~ (agonist) of low -molecular -weight substances (antagonist) exhibiting the ~~having an~~ EPO-like activity were synthesized through synthesis of organic chemical compounds based on the three-dimensional structure has already been successful (Nicholas *et al.*: Science, 273, 458- to 463, 1996).

~~The present~~ Furthermore, the inventors have ~~previously discovered using the osteoclastogenesis inhibitory factor, OCIF, found that an OCIF-binding a protein binding~~ OCIF is specifically expressed on ~~osteoblastic~~ an osteoblast-like stromal cell line, ST2, which ~~was~~ cultured in the presence of ~~a osteotropic bone resorption factors~~ such as the

active-form of vitamin D₃ and parathyroid hormone (PTH), using osteoclastogenesis inhibitory factor (OCIF). Moreover, the inventors further have found that this protein exhibits a biological activity to support or stimulate, which is associated with differentiation or maturation of osteoclasts from immature osteoclast precursor cells, and clarified various to osteoclasts and maturation thereof, has a biological activity as a factor which supports and promotes so-called differentiation and maturation of osteoclasts. After purification of the protein, the physicochemical properties and the biological activity of this protein by purification thereof, the protein were examined. In order to compare the inventors have compared the physicochemical properties and biological activity of the recombinant protein OBM expressed by expressing the DNA of the present invention and with those above mentioned of a purified natural -type protein which specifically binds to OCIF, the present inventors investigated the physicochemical properties and biological activities of the two proteins in order to clarify differences between them. As a result, the two proteins were confirmed ① to be they have found that (1) each of both proteins is a membrane-bound proteins which and specifically bind to binds OCIF, ② to have; (2) they shared a molecular weights of approximately about 40,000 determined as measured by SDS-PAGE; and ③(3) to they have an apparent molecular weights of about 90,000- to 110,000 when cross-linked crosslinked to with a monomer-form-type OCIF. Not only are these, which indicates that they have very similar physicochemical properties identical, but both proteins exhibit a biological. An activity to support and stimulate promote differentiation and maturation of osteoclasts was also shared by them as well. Therefore, suggesting the possibility that these both proteins are the same protein identical was suggested. In addition Furthermore, a rabbit anti-OBM rabbit polyclonal antibody produced using the purified protein prepared by expressing with the protein (recombinant OBM), which was genetically expressed with the DNA of the present invention by a genetic engineering technique (recombinant OBM) was confirmed to and then purified, has cross-react with the above described reactivity to the purified natural type protein, to inhibit specific-type protein obtained by the above method and specifically inhibited the binding of this purified between said natural -type protein and OCIF in the same manner, just as the antibody it inhibits specific binding of between OBM and OCIF. Based on From these results, it is clear obvious that the

recombinant protein OBM expressed by with the DNA of the present invention is identical to the natural γ -type protein which specifically binds to OCIF.

~~To isolate~~ Furthermore, for isolating a gene (cDNA) ~~encoding that encodes a~~ human-derived OCIF-binding protein molecule (hereinafter ~~called~~ referred to as "human OBM") which specifically binds to OCIF and ~~exhibits~~ has the activity to support and stimulate ~~promote~~ differentiation and maturation of osteoclasts from mouse spleen cells in the same manner to osteoclasts and maturation, just as the natural γ -type or the recombinant mouse OBM does, a cDNA library prepared from mRNA derived from human lymph nodes was screened using a human OBM cDNA fragment as a probe. The human OBM cDNA fragment was obtained by ~~does~~, the inventors have carried out a polymerase chain reaction (PCR) in accordance with the method mentioned using primers prepared based on the above using both mouse OBM cDNA prepared from and human lymph node-derived cDNA as a template and the primer which was prepared from mouse. Thus, the inventors have screened said cDNA library with the obtained human OBM cDNA cDNA fragment. As a result, they have succeeded in isolation of the cDNA ~~encoding which encodes~~ the human-derived protein which specifically binds to OCIF was (human isolated OBM) and ~~determination of~~ the nucleotide sequence of the said cDNA ~~encoding this human OCIF-binding protein molecule (i.e. the cDNA encoding human OBM) was determined~~. Similar to mouse OBM, this They have found that human OBM encoded by the cDNA has characteristics to bind to OCIF strongly and specifically binds OCIF on the cell membrane and ~~exhibits the~~ has a biological activity to support and promote differentiation and maturation of osteoclasts from mouse spleen cells to osteoclasts and maturation thereof, just as mouse OBM does. Specifically That is, other objects of the present invention provides are to provide: (1) DNA ~~encoding which encodes human OBM which is a novel human OBM-derived protein which binds to osteoclastogenesis inhibitory factor (OCIF);~~ a protein which possesses the having an amino acid sequence encoded by the DNA; (2) a method for genetically producing the protein exhibiting characteristics of which specifically binding to binds OCIF and the has an activity to support and promote differentiation and maturation of osteoclasts from mouse spleen cells to osteoclasts and maturation thereof by genetic engineering techniques, pharmaceutical compositions comprising this protein use of the DNA; (3) an

agent for the treatment of diseases caused by abnormality of treating bone metabolism; abnormality comprising the protein; (4) a method for screening substances regulating a substance which controls expression of human OBM; (5) a method for screening substances a substance which inhibit binds human OBM and inhibits or modulate the activity of human OBM by binding to it, modifies an effect thereof; (6) a method for screening receptors a receptor which bind to binds human OBM and transmit the action of OBM, transmits an effect thereof; and (7) a pharmaceutical compositions comprising the substances obtained by as a result of these screenings methods for screening.

The present invention further provides relates to DNA encoding which encodes human OBM, a novel human OBM-protein, which specifically binds to OCIF and exhibits has the biological activity to support and promote differentiation and maturation of osteoclasts; a protein which possesses the having an amino acid sequence encoded by the DNA; a method for genetically producing the protein exhibiting characteristics of which specifically binding to binds OCIF and the has an activity to support and promote differentiation and maturation of osteoclasts by genetic engineering techniques, with the DNA; and pharmaceutical compositions comprising this protein an agent for the treatment of diseases causing abnormality of treating bone metabolism abnormality comprising the protein. Furthermore, the present invention provides also relates to a method for screening substances regulating a substance which controls expression of human OBM; a method for screening substances a substance which inhibit binds human OBM and inhibits or modulate the activity of human OBM by binding to it, a modifies an effect thereof; a method for screening receptors binding to human OBM and transmitting the action of OBM, antibodies against a receptor which binds human OCIF binding protein, OBM and transmits a biological activity of OBM; a pharmaceutical compositions comprising a substance obtained as a result of these antibodies methods for screening; an antibody to the prevention human-derived OCIF binding protein; and an agent for preventing and/or treatment of diseases causing abnormality of treating bone metabolism abnormality using the antibody.

The novel, human-derived OCIF- binding protein molecule-(, human OBM) which is, encoded by the DNA of the present invention has shows the following physicochemical properties and biological activity. That is, (a) binds human OBM

specifically binds to osteoclastogenesis inhibitory factor (OCIF) (WO 96/26217); (b) has human OBM shows a molecular weight of approximately about 40,000 ($\pm 5,000$) when as determined measured by SDS-PAGE under reducing conditions and shows an apparent molecular weight of approximately about 90,000- to 110,000 when crosslinked with a monomer-form-type OCIF; and (c) exhibits human OBM has a biological activity to support and stimulate promote differentiation and maturation of osteoclasts.

Mouse OBM The cDNA which ~~encodes~~ encoding mouse OBM, mouse-derived OCIF- binding protein ~~and used, useful~~ as a probe ~~to for~~ isolateseparating and identifying the cDNA ~~encoding which encodes~~ human OBM of the present invention, can be isolated ~~according to the above mentioned method~~ from a cDNA library of a mouse osteoblasticosteoblast-like stromal cell line, ST2. Human Furthermore, human osteoclastogenesis inhibitory factor (OCIF) ~~which is necessary, required~~ to evaluateexamine the properties and the biological activity of the protein obtained by ~~expression of~~ expressing human OBM cDNA, can be ~~prepared according to the method described in WO 96/26217 by isolatingisolated~~ from the culture brothsolution of human fibroblast cell line, IMPstrain IMR-90,90 in accordance with the method described in WO 96/26217, or by genetic engineering techniques using it can be genetically produced with the DNA encoding OCIF it. Recombinant human OCIF To examine the properties and biological activity of human OBM, recombinant humOCIF, recombinant mouse OCIF, recombinant rat OCIF, ~~or and~~ the like can also be ~~used for the assessment of the properties and biological activity of human OBM~~. These recombinant OCIFs can be obtained ~~according to conventional methods by inserting cDNA incorporating the corresponding cDNAs into an expression vectorvectors~~ in accordance with a commonly used method, expressing the cDNAOCIFs in animal or insect cells such as CHO cells, BHK cells, ~~or and~~ Namalwa cells, ~~or in insect cells~~, and purifying the expressed proteins them.

~~The following methods can be used to isolate~~ Methods for isolating the human cDNA ~~encoding which encodes~~ the target protein (cDNA cloning): include: (1) Aa method comprising the steps of purifying the protein, determining ~~thea~~ a partial amino acid sequence ~~of the protein thereof~~, and isolating the target cDNA by a hybridization using ~~the with~~ DNA fragmenthaving comprisinga nucleotide sequence corresponding to the

amino acid sequence as a probe, ② (2) a method ~~applied even in the case where the amino acid sequence (expression cloning method) comprising the steps of the protein is not known, which comprises~~ constructing a cDNA library ~~in with an~~ expression vector, regardless of whether the amino acid sequence of the target protein is unknown, introducing the ~~cDNA library~~ them into cells, and screening for the presence and absence of the expression of the target protein so as to isolate the objective target cDNA (expression cloning method);; and ③ (3) a method of isolating the cDNA encoding which encodes the target human protein from by the hybridization or polymerase chain reaction (PCR) method from cDNA library constructed using from human cells or tissues by hybridization tissue or by the use of polymerase chain reaction (PCR) using the cDNA encoding the which encodes a protein of mammalian origin (derived from a mammal other than human) which possesses and having the same characteristics properties and biological activity as the of the human-derived target protein of human origin as a probe, assuming that the cDNA probe has, based on an assumption that the cDNA which encodes the non-human protein shares high homology with the human origin cDNA which that which encodes the desired corresponding human protein to be cloned.

Based on ~~the assumption that human OBM cDNA has a high homology with an~~ assumption that human OBM cDNA is highly homologous with the above mouse OBM cDNA, it is possible to determine which human cells or tissues produceing human OBM can be identified by Northern hybridization method using the latter (mouse OBM) cDNA as a probe. Human OBM cDNA can be cloned as follows. A human OBM cDNA fragment is obtained by the following method using the through PCR using mouse OBM primer prepared from the primers prepared based on the mouse OBM cDNA and the cDNA. Human OBM cDNA fragments can be prepared by the PCR method using cDNA prepared from library of a cell or tissue which produces human OBM-producing tissues such as (e.g., a human lymph nodes node) as identified above, as primers and a template, respectively. These human OBM cDNA fragments are used as probes for screening the The cDNA library of human OBM-producing cells or tissues which were produce human OBM as identified according to the method mentioned above is screened with the human OBM cDNA fragment as a probe, and thus, human OBM cDNA can be obtained. The present invention relates to the DNA encoding human OBM which has

~~characteristics of specific binding to~~obtained DNA that encodes human OBM, a human-derived protein which specifically binds OCIF and ~~exhibits~~has biological activity to support and promote differentiation and maturation of osteoclasts. ~~Because~~Since the human OBM ~~which is encoded by the DNA of the present invention is a~~ membrane-bound type-protein which comprises having a transmembrane domain, ~~this protein~~ it can be detected by labeling OCIF and ~~by examining the~~then binding of the labeled OCIF to the surface of an animal cells in which the cDNA of the present invention ~~was~~is expressed. ~~The above described~~In such a case, OCIF can be labeled by a method which is conventionally used for labeling method using protein such as labeling with a radioisotope or fluoresceine conventionally applied to and fluorescence labeling proteins can be used for labeling OCIF.

The molecular weight of the protein expressed by ~~the~~ human OBM cDNA of the present invention ~~can be accessed~~is determined by gel filtration chromatography, SDS-PAGE, ~~or and~~ the like. ~~In order to accurately~~To determine the molecular weight more accurately, ~~it is desirable to use the SDS-PAGE method~~is preferably used, by which ~~and~~ human OBM ~~was~~is ~~specified~~identified as a protein having a molecular weight of approximately about 40,000 (40,000 \pm 5,000) under reducing conditions.

~~Comparatively~~An example of DNA hybridization under relatively mild conditions for hybridization of DNA in the present invention are the conditions, for example, wherein is that after DNA is transferred to a nylon membrane and immobilized there to fixed in accordance to with a conventional commonly used method and, the DNA is hybridized with another radiolabeled DNA as a probe DNA labeled with an isotope in a buffer solution for hybridization buffer at a temperature of 40-° to 70°C for about 2 hours to overnight, followed by washing in and then washed with 0.5 \times SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 45°C for 10 minutes. Specifically More specifically, Hybrid after DNA is transferred and fixed to a nylon membrane, which is HYBOND® N (Amersham Co., Ltd.) is used as, in accordance with a conventional method, the nylon membrane to transfer and immobilize DNA thereon. The DNA is then DNA is hybridized with another 32 P-labeled DNA as a probe DNA labeled with 32 P in a rapid hybridization buffer Rapid Hybridization Buffer (Amersham Co., Ltd.) at 65°C

for 2 hours, followed by and then washing with the above 0.5-X SSC at 45°C for 10 minutes.

~~A~~As a representative *in vitro* culture system for osteoclastogenesis, a co-culture system of mouse-derived osteoblastic osteoblast-like stromal cell line, ST2, and mouse spleen cells in the presence of the active-form of vitamin D₃ or PTH is well known as a typical *in vitro* culture system for osteoclast formation. ~~Interaction by adhesion of osteoblastic~~ For promoting osteoclastogenesis in this *in vitro* culture system, both the interaction between a osteoblast-like stromal cells and a spleen cell through their binding, and the presence of an osteotropic bone resorption factors such as the active-form of vitamin D₃ ~~or~~ PTH are indispensable for the osteoclasts formation in this *in vitro* culture system essential. In this *in vitro* culture system, a recombinant COS cells, monkey kidney cells having no osteoclast formation supporting capability, acquire capability to support osteoclasts formation cell strain, resulting from spleen cells in the absence of an osteotropic factor when the expression of the cDNA of the present invention was expressed as osteoblastic thereon, has obtained an ability to support osteoclast formation from spleen cells, just like the osteoblast-like stromal cell line ST2 did, while COS-7 cells (a monkey kidney-derived cell line) does not have an ability to support osteoclast formation in the absence of said bone resorption factors. ~~Based on the fact that~~ Furthermore, since the cDNA of the present invention encodes a membrane-bound protein comprising a transmembrane domain form, this cDNA protein can be expressed as a secretionary form type or solubilized form by type protein after removing the part fragment which encodes this transmembrane the membrane binding domain thereof. It ~~was~~ has also been confirmed that osteoclastogenesis ~~can be formed was promoted simply~~ by adding the addition of the secretion form secretory-type human OBM to the above-mentioned *in vitro* culture system in the absence of ~~osteotropiesaid bone resorption~~ factors. ~~Based on~~ From these results, the human OBM ~~which is encoded by the cDNA of the present invention is specified identified as the~~ factor involved in the differentiation and maturation of osteoclasts.

~~A recombinant~~ Recombinant human OBM can be prepared by inserting the cDNA of the present invention into an expression vector, ~~preparing so as to prepare a plasmid for expressing human OBM-expression plasmid, and then~~ introducing and

expressing the plasmid in various cell cells and strains and expressing OBM in the cells. Mammalian cells such as COS-7, CHO, and Namalwa cells, or bacteria such and the like can be used as Escherichia mammalian host cells suitable for expression, and E. coli and the like can be used as a bacterial host for expressing OBM cells. In those cases, recombinant human OBM may can be expressed as a membrane-bound-form protein, by using the full length of DNA, or as a secretory-form type or solubilized-form type protein by removing a region which encodes the part encoding the transmembrane membrane binding domain. The Thus produced recombinant human OBM thus produced can be purified efficiently purified using a suitable in combination of with conventionally purification used methods used for common purifying proteins such as affinity chromatography using OCIF -immobilized column or a column, ion exchange chromatography, and gel filtration chromatography and the like. Human Thus obtained human OBM of the present invention thus obtained is useful, due to its activity, as a medicament, e.g., as an agent for treating diseases caused by abnormality of bone metabolism abnormality such as osteopetrosis or as an experimental and diagnostic reagent for research and diagnosis of such diseases.

The following screening operations can be carried out using the human OBM protein OBM encoded by the DNA DNA of the present invention enables: (1) screening of substance a substance which can regulate controls expression of human OBM₁; (2) screening of substance a substance which specifically bind to binds human OBM and inhibits or modifies the biological activity of human OBM₁; and (3) screening of a human proteins protein (human OBM receptor) which are present exists in osteoclast precursor cell cell of human osteoclasts and transmits the biological activity of human OBM (human OBM receptor). It is also possible to develop antagonists, as well as development of antagonist and agonists using this human OBM receptor. In the combinatorial chemistry using the above human OBM or human OBM receptor, a peptide libraries required library, which is employed for the screening identification of antagonists an antagonist or agonists agonist, can be produced by prepared in accordance with the same method as used for the screening using the mouse OBM. A After screening the peptide with extremely library by said method in which human OBM is used instead

of mouse OBM, a specific peptide having very high specificity and affinity can be obtained by screening peptide libraries using human OBM instead of mouse OBM.

Although this OBM is very Furthermore, for measurement of OBM, a highly useful as protein mentioned described above and antibodies, it is necessary to obtain an antibody which specifically recognizes OBM and establish an enzyme immunoassay using these antibodies are indispensable in determination of OBM concentration it. However, no antibodies useful for the access measurement of OBM concentration have has been so far available. In addition Moreover, an anti-OBM antibody or anti-/sOBM antibody which neutralizes the biological activity of OBM or sOBM is supposed assumed to suppress the an activity of OBM or sOBM, specifically the activity to induce promote osteoclasts formation. These, are and expected to be useful developed as therapeutic agents to treat abnormality of an agent for treating bone metabolism abnormality. However, no such antibodies have so far an antibody has not been available.

In view of this situation Under that circumstance, the present inventors have conducted extensive made intensive studies. As a result, the present inventors and have found antibodies (anti-OBM/sOBM antibodies) which recognize both OBM of the following antigens, a membrane-bound protein (OCIF binding molecule; OBM) which specifically binds to osteoclastogenesis inhibitory factor (OCIF); and a soluble-type OBM (sOBM) which lack a transmembrane lacking the membrane binding domain. Accordingly Therefore, objects of the present invention provides antibodies are to provide: (1) an antibody (anti-OBM/sOBM antibodies) which recognizes both OBM of the following antigens, a membrane-bound protein (OBM) which specifically binds to osteoclastogenesis inhibitory factor (OCIF); and soluble OBM (sOBM which lack a transmembrane) lacking the membrane binding domain; (2) a method for the preparation production thereof; (3) a method for determination of measuring OBM and the sOBM concentrations using these antibodies by use of said antibody; and agents (4) an agent for the prevention preventing and/or treatment of diseases resulting from abnormality of treating bone metabolism abnormality which comprises said antibody as an active ingredient.

The present invention relates to antibodies: (1) an antibody (anti-OBM/sOBM antibodies) which recognizes both of the OBM following antigens, a membrane-bound

protein (OCIF binding molecule; OBM) which specifically binds to osteoclastogenesis inhibitory factor (OCIF); and a soluble-type OBM (sOBM) which lack a transmembrane lacking the membrane binding domain; (2) a method for the preparation/production thereof; (3) a method for quantifying/measuring OBM and the sOBM using these antibodies by use of said antibody; and agents (4) a pharmaceutical composition comprising said antibody as an active ingredient, particularly, an agent for the prevention/preventing and/or treatment of diseases resulting from abnormality of treating bone metabolism abnormality.—The antibodies

An antibody of the present invention exhibits an antibody which has an activity of neutralizing the to neutralize osteoclastogenesis accelerating/promoting activity, which is the a biological activity that OBM and sOBM have, said antibody has any of OBM and sOBM and comprises the antibodies having the following characteristics/properties: (a) a polyclonal antibody which recognizes both mouse OBM and mouse sOBM antigens (anti-mouse OBM/sOBM polyclonal antibody); (b) a polyclonal antibody which recognizes both human OBM and human sOBM antigens (anti-human OBM/sOBM polyclonal antibody); (c) a monoclonal antibodiesy which recognizes both mouse OBM and mouse sOBM antigens (anti-mouse OBM/sOBM monoclonal/polyclonal antibodiesy); (d) a monoclonal antibodiesy which recognizes both human OBM and human sOBM antigens (anti-human OBM/sOBM monoclonal/polyclonal antibodiesy);; and (e) an anti-human OBM/sOBM monoclonal antibodiesy which crossreacts has crossreactivity to both mouse OBM and mouse sOBM antigens.

The polyclonal antibody which recognizes both mouse OBM and mouse sOBM antigens (hereinafter called-referred to as “anti-mouse OBM/sOBM polyclonal antibody”) and the polyclonal antibody which recognizes both human OBM and human sOBM antigens (hereinafter called-referred to as “anti-human OBM/sOBM polyclonal antibody”) were produced/can be obtained by the following method/means. The A purified mouse OBM used as an antigen for immunization can be obtained according to in accordance with the above-mentioned method. Especially That is, natural-type mouse osteoblastic OBM can be obtained by treating a mouse osteoblast-like stromal cell line, ST2, was treated with the active-form of vitamin D₃ and OBM on subsequently purifying it from the cell membrane was purified using membranes of said cell by

means of OCIF- immobilized on a column and gel filtration chromatography, thereby obtaining natural mouse OBM (native OBM). Alternatively, after incorporating the above-mentioned mouse OBM cDNA (Sequence Table, Sequence No. SEQ ID NO: 15) or human OBM cDNA (Sequence Table, Sequence SEQ ID No. NO: 12) was inserted into an expression vector according to conventional methods. Recombinant mouse OBM (Sequence Table, Sequence ID No. 1) and recombinant human OBM (Sequence Table, Sequence ID No. 11) can be obtained by expressing cDNA OBM in an animal cell or insect cell such as a CHO cells, a BHK cells, Namalwa, or a COS-7 cells, insect cells or *Escherichia-E. coli*; and then purifying them using by the same purification methods method as mentioned described above. These recombinant mouse OBM (SEQ ID NO: 1) or recombinant human OBM (SEQ ID NO: 11) can be obtained, and these may also be used as antigens for immunization. In At this instance time, purifying it takes tremendous effort to highly purify a large amount and a high level quantity of mouse OBM or human OBM which are, a membrane-bound proteins is a task requiring a great deal of labor protein (OBM). On the other hand, as mentioned above, OBM which it has been confirmed that there is no difference in ability to promote differentiation and maturation of osteoclasts between OBM, a membrane-bound protein, and a soluble type OBM (sOBM), which is a soluble protein obtained by deleting transmembrane the membrane binding domain of OBM are known to be almost the same in their osteoclast differentiation and maturation activities as described above. It is possible to use Accordingly, taking into account that expression and high purification of mouse sOBM and human sOBM which are relatively easily expressed and purified to a high level easy, these sOBMs, solubilized proteins, may be used as antigens for immunization. Mouse sOBM (Sequence Table, Sequence SEQ ID No. NO: 16) and human sOBM (Sequence Table, Sequence SEQ ID No. NO: 17) can be obtained by adding a nucleotide sequence encoding, which encodes a known signal sequence originating derived from the other secretion protein in these secretory-type proteins, 5' upstream side of the 5' end of, respectively, mouse sOBM cDNA (Sequence Table, Sequence SEQ ID No. NO: 18) and or the human sOBM cDNA (Sequence Table, Sequence SEQ ID No. NO: 19), inserting these incorporating the cDNA into an expression vector by the use of genetic in accordance with the same gene engineering techniques, causing these proteins to be

~~expressed in host cells such as various method as described above, expressing the protein in a variety of animal cells, insect cells, or Escherichia coli as a host, and then purifying the resultant products. The antigens thus obtained antigen for immunization thus obtained are~~ dissolved in a phosphate buffered saline solution (PBS) and, if necessary, mixed with ~~the an same equal~~ volume of Freund's complete adjuvant ~~to emulsify and emulsified~~. Then, an animal is immunized with the solution ~~if required, and subcutaneously administered to animals about once emulsion through a week to immunize these animals several few times of subcutaneous administration with a one-week interval between each. A booster injection is given when the~~ The antibody titer is measured. When the value reaches a maximum. ~~Exsanguination, booster administration is performed. On the 10 days afterth day from the booster administration, all the blood was collected. The resulting obtained antiserum is treated fractionated and precipitated with ammonium sulfate precipitation. IgG, and the globulin fraction is purified using with an anion exchange chromatography or the antiserum is diluted twice with Binding Buffer (Bio-Rad Co., Ltd.) and the diluted antiserum is purified by pProtein A- or pProtein G-Sepharese SEPHAROSE® (Pharmacia Co., Ltd.) column chromatography after diluting the antiserum two fold with Binding Buffer™ (BioRad Co.) Thereby, to obtain the desired anti-mouse or anti-human OBM/sOBM polyclonal antibody can be obtained.~~

The monoclonal antibodies of the present invention can be obtained according to the following method. ~~In the same manner~~ That is, as in the case of the polyclonal antibodies, ~~an antigen for immunization required to prepare the monoclonal antibody, a natural type mouse OBM (native OBM), recombinant mouse or human OBM, or recombinant mouse or human sOBM can be used, as immunogens to prepare monoclonal antibodies used in preparation of the above polyclonal antibody. Hybridomas are produced according to conventional methods by immunizing Lymphocytes derived from immunized mammals with these each antigens or that obtained by immunizing lymphocytes in vitro and fusing the immunized cells methods are fused with a myeloma cell line, and hybridomas are prepared in accordance with a conventional method. By analyzing~~ From the hybridoma culture supernatant thus obtained of this hybridoma, a hybridoma producing an antibody which recognizes each antigen is selected by a solid - phase ELISA method, antibody-producing hybridomas recognizing the, using each highly

purified antigen are selected. The resulting obtained hybridomas are cloned, and established as thus obtained stable antibody-producing hybridoma clones. These hybridomas are cultured to obtain the target antibody can be obtained therefrom. For preparation of the antibodies. Small mammal hybridoma, immunizing a mammal, a small animal such as mice or rats, are commonly used to produce hybridomas. Animals are immunized by intravenously or intraperitoneally injecting the antigen diluted to a suitable concentration using a suitable solvent. To immunize the animal, a method comprising the following steps is conventionally used: diluting the antigen with an appropriate solvent such as physiological saline solution. Optionally to an appropriate concentration and then administering the solution and, if necessary, co-administering Freund's complete adjuvant may be used together with antigen, into vein (i.v.) or the abdominal cavity (i.p.), about 3- or 4 times, once in total with a 1 to 2-week or every two weeks interval between each. The immunized animals are dissected three days on the 3rd day after final the last immunization. Splenocytes, and spleen cells are obtained from the removed isolated spleen and used as immunocytes (immunized cells). As illustrative examples of mouse-derived myeloma to be for cell fusion with immunized cells, the immunocytes include p3/x63-Ag8, p3-U1, NS-1, MPC-11, SP-2/0, F0, F0, P3x63 Ag8.653, 8.653 and S194 can be given. 194. Furthermore, illustrative examples of rat-derived cells include cell lines such as R-210 is given as the cell of rat origin. 210. Human antibodies are produced by immunizing. For producing human antibody, human B lymphocyte cells are immunized *in vitro* and fusing the immunized cells fused with human myeloma cells or a cell line transformed with EB virus. The fusion of the immunized cells and cell with a myeloma cells can be carried out cell line is performed according to a conventional known methods such as the method that of Kohler and Milstein *et al.* (Kohler *et al.*: Nature 256, 495-497 (to 497, 1975)). A, while an electric pulse method using an electric pulse is may also applicable be used. Immunized lymphocyte cells and myeloma cell lines are mixed together at a ratio conventionally accepted ratio used and fused in an FCS-free (fetal common bovine fetal serum (FCS)-free) medium for cell culture medium with an addition of in which polyethylene glycol is added. Then, and cultured culture is carried out in an FCS-containing HAT selection medium so as to

select a fused cells (hybridomas). Next, the hybridomas which produce producing an antibodies are selected by using a conventional antibody detection commonly used method for detecting antibody such as an ELISA, a plaque technique, Ouchterlony method, Ouchterlony method or aggregationcondensation method. Thereafter, to establish stable hybridomas a hybridoma is established. The hybridomas established in this way hybridoma can be subcultured by a conventional common method for culture method or and can be stored by freezing as required in a frozen state if necessary. A The hybridoma can may be cultured by in accordance with a conventional conventionally used method to collect the culture supernatant or implantedtransplanted in the abdominal cavity of mammals to obtain the mammal. The antibody can be collected from the ascitic fluidresulting culture solution or ascites, respectively. The antibody in the culture supernatantsolution or ascitic fluidascites can be purified by a conventional commonly used method such as a salting out method, ion exchange andchromatography, gel filtration chromatography, or pProtein A or pProtein G affinity chromatography. Almost all the monoclonal antibodies obtained by the above-described method using sOBM as an antigen are antibodies which can specifically recognize not only sOBM but also OBM (such antibodies are hereinafter calledreferred to as "anti-OBM/sOBM monoclonal antibodiesantibody"). These antibodies can be used for the quantification of OBM or sOBM. The amountsmeasurements of OBM and sOBM can be quantified by labeling. After these antibodies are labeled with a radioisotope or an enzyme and by applying the labeled antibodies thus employed to a quantification systemmeasurement systems known such as aas radioimmunoassay (RIA) or enzymeimmunoassayand enzyme immunoassay (EIA), an amount of OBM and sOBM can be measured thereby. UsingBy use of these quantificationmeasurement systems, thean amount of sOBM in a biologicalliving sample such as blood or urine can be determinedmeasured with ease atand with high sensitivity. In addition, theFurthermore, by use of these antibodies, an amount of OBM bindingbound to the surface of a tissue or surface of cellscell can be measured through a binding assay or the like with ease atand with high sensitivity utilizing a binding assay using these antibodies.

When anthe obtained antibody is used as a medicamentio for humans, it is desirable to use, in consideration of a problem of antigenicity, that a human-type anti-

human OBM/sOBM antibody ~~in view of antigenicity is prepared~~. The human-type anti-human OBM/sOBM antibody can be prepared ~~according to~~ the following methods ①, ②, or ③. ~~In the method ①~~ That is, (1) human lymphocytes ~~collected lymphocyte cells~~ extracted from human peripheral blood or the spleen are ~~immunized~~ sensitized *in vitro* with an antigen-human OBM or human sOBM ~~in vitro as an antigen~~ in the presence of IL-4. The resulting immunized ~~4~~, and then the sensitized human lymphocytes ~~lymphocyte cells~~ are fused with K₆H₆/B₅ (ATCC CRL1823), which is a hetero -hybridoma of mouse and human, and thereby, a hybridoma producing the desired antibody is screened ~~to obtain the objective~~. An antibody-producing hybridoma. The antibodies produced by the resulting antibody-producing hybridomas are ~~from the obtained hybridoma is a human -~~ type anti-human OBM/sOBM monoclonal antibodiesy. The antibodies neutralizing ~~Among these antibodies, an antibody which neutralizes the activity of human OBM/sOBM are is selected from these antibodies~~. However, in general, it is usually difficult to ~~produce obtain~~ an antibody-exhibiting ~~having high affinity to for an antigen by the through such a method of immunizing sensitizing human lymphocytes lymphocyte cells in vitro~~. Therefore, ~~in order to obtain for obtaining a monoclonal antibodiesy with having high affinity to for human OBM and sOBM, it is necessary to increase the affinity of the human type modify an anti-human OBM/sOBM monoclonal antibodies obtained by the antibody with low affinity as described above method~~. This ~~can to be done according to the following method that with high affinity~~. First, aA random mutation is introduced into a CDR region (particularly CDR-3 region ~~in particular~~) of a said human-type anti-human OBM/sOBM monoclonal antibody ~~which neutralize OBM but have a with low affinity, and make the which a neutralizing antibody obtained as described above~~. This is expressed with phage ~~to express protein~~. Phages which ~~can strongly bind to human OBM/sOBM which has the antigen~~ are selected by a phage display method using plates on a plate in which human OBM/sOBM antigens ~~are is~~ immobilized. The selected phages are grown ~~phage is allowed to proliferate in Escherichia E. coli~~. The, and the deduced amino acid sequence of the CDR ~~which exhibits having high affinity is determined from based on the nucleotide sequence of the DNA cloned in the phage thereof~~. The thus-obtained DNA-encoding gene which encodes the human -type anti-human OBM/sOBM monoclonal antibodiesy is introduced ~~into incorporated and expressed in a~~

commonly conventionally used expression vector for mammalian cells to produce, and then human-type anti-human OBM/sOBM monoclonal antibodies can be obtained.
Among them, the desired human -type anti-human OBM/sOBM monoclonal antibodies.
The target human-type anti-human OBM/sOBM monoclonal antibodies exhibiting high affinity and capable of neutralizing antibody which neutralizes the biological activity of human OBM/sOBM and has high affinity thereto can be selected from these monoclonal antibodies.
In the method ② Furthermore, mouse-type anti-human OBM/sOBM monoclonal antibodies are produced according to the same method as in the present invention(2) using BALB/c mouse, an anti-human OBM/sOBM mouse monoclonal antibody is prepared according to a conventionally used method (Koehler *et al.*: Nature 256, 495-49, to 497, 1975) as in the present invention, and a monoclonal antibodiesy which can neutralize neutralizes the biological activity of human OBM/sOBM and exhibiting has high affinity are thereto is selected. These high affinity mouse anti-human OBM/sOBM monoclonal antibodies can be converted into human-type using the By CDR- grafting technique method (Winter and Milstein: Nature 349, 293- to 299, 1991) by implanting its, that is a method in which a CDR regions (CDR-1, 2 and 3) of the anti-human OBM/sOBM mouse monoclonal antibody with high affinity are transplanted into the CDR regions of human IgG, a humanized antibody can be obtained. In the method ③ Moreover, (3) human peripheral blood lymphocytes lymphocyte cells are implanted transplanted into a severe combined immune deficiency (SCID) mouse. Because the implanted Thus transplanted SCID mouse can produce produce a human antibodiesy (Mosier D. E. *et al.*: Nature 335, 256- to 259, 1988; Duchosal M. A. *et al.*: Nature 355, 258- to 262, 1992), lymphocytes which can produce the human monoclonal antibodies having specificity to human OBM/sOBM can be collected by screening SCID mouse immunized. The cells are sensitized with human OBM or sOBM as an antigen and screened. The resulting lymphocytes Thereafter, a lymphocyte cell which produces a human-type monoclonal antibody specific to human OBM/sOBM can be extracted from the mouse. Then, as in the case of the above method for preparing a human-type antibody (1), the obtained lymphocyte cells are fused with K₆H₆/B₅ (ATCC CRL1823) which is a heterohybridoma, a hetero hybridoma of mouse and human, according to and then the obtained hybridomas are screened. Then, a hybridoma which

produces the target human-type monoclonal antibody can be obtained. By culturing the thus obtained hybridoma, the procedure described above for the target human antibodies in the method ①. The resulting hybridomas are screened to obtain hybridomas which can produce the objective human-type monoclonal antibodies. The thus obtained hybridomas are cultured to produce antibody can be produced in large quantities. After purifying them in the same manner as described above, large amounts of the objective human monoclonal antibodies. The antibodies pure products thereof can be purified by the above mentioned purification method obtained. In addition, it is possible to produce Furthermore, a recombinant human-type monoclonal antibodies antibody can be produced in large amounts quantities by constructing a cDNA library from the said hybridoma which can produce produces the objective human monoclonal antibodies to obtain a gene (cDNA) encoding the objective target human-type monoclonal antibodies by antibody, cloning the cDNA which encodes the target human-type monoclonal antibody, in incorporating this gene said cDNA into an suitable appropriate expression vector by using genetic engineering techniques gene engineering, and expressing the monoclonal antibodies antibody in host cells such as various a variety of animal cells, insect cells, or *Escherichia coli* as a host. A large amounts of purified human monoclonal antibodies can be obtained by purifying from the resulting After purification of the antibody from said culture supernatant by the purification methods mentioned above according to the method as described above, a large amount of pure human-type monoclonal antibody can be obtained.

The antibodies which can neutralize Among the anti-OBM/sOBM monoclonal antibodies obtained by the above method, moreover, an antibody which neutralizes the biological activity of OBM/sOBM can be obtained from the anti-OBM/sOBM monoclonal antibodies produced according to this method. The These antibodies which neutralize the biological activity of OBM/sOBM are expected to be useful as medicaments, particularly agents for the treatment preventing and/or prevention of treating bone metabolism abnormality because of their capability of blocking in vivo, since they can inhibit the biological action (an activity of OBM/sOBM, specifically the capability of preventing the induction to promote osteoclast formation) of OBM/sOBM in a living body. The activity of the anti-OBM/sOBM antibodies to neutralize the

biological activity of OBM or sOBM can be measured by determining the determined as an activity to ~~suppress~~inhibit osteoclast formation in the ~~an~~ *in vitro* system for examining osteoclast formation. Specifically ~~As in vitro assay systems~~, the following three methods can be used. That is, *in vitro* culture systems for examining osteoclastogenesis culture system ~~can be given~~include: ① (1) a co-culture system of a mouse osteoblastic~~osteoblast-like~~ stromal cell ~~strainline~~, ST2-cells~~2~~, and mouse spleen cells in the presence of the active-form of vitamin D₃ and dexamethasone;; ②(2) a co-culture system ~~comprising~~in which OBM ~~expressing~~is expressed on a monkey kidney cell ~~strainline~~, COS-7, ~~immobilizing the OBM-expressing cells and fixed~~ with formaldehyde, and ~~culturing mouse spleen cells on those~~then mouse spleen cells are cultured on the cells in the presence of M-CSF; and ③ a culture system of mouse spleen cells; and (3) a system of culturing mouse spleen cell in the presence of recombinant sOBM and M-CSF. ~~The~~ however, other systems can be also used. When an anti-OBM/sOBM antibody is added to such a culture system in various concentration and its effect on osteoclastogenesis-inhibitory is examined, ~~an activity of the anti-OBM/sOBM antibodies can be measured by adding the anti~~ of the anti-OBM/sOBM antibodies at various concentrations to these culture systems and investigating their effects on osteoclast formation. ~~The antibody to inhibit osteoclastogenesis-inhibitory can be measured~~. Also, the activity ~~of~~ of the anti-OBM/sOBM antibodies can also be evaluated by measuring their OBM/sOBM antibody to inhibit osteoclastogenesis can be determined as an activity to suppress bone resorption-inhibitory activity utilizing in vivo using an experimental animals in vivo. Especially, ~~ovariectomized animal model is given as an~~. That is, there is an animal model with progressive osteoclast formation. ~~The osteoclastogenesis-inhibitory activity of the anti-OBM/sOBM antibodies can be determined by administering the~~, an overiectomized mouse, in which osteoclastogenesis is increased. ~~An anti-OBM/sOBM antibodies to such -OBM/sOBM antibody is administered to such a kind of experimental animals and evaluating the suppression of animal~~, and an activity to inhibit bone resorption-(a (an activity to reinforce bone mineral density-increasing activity) is measured. Thereby, an activity of the anti-OBM/sOBM antibody to inhibit osteoclastogenesis can be determined.

~~The thus-obtained antibodies capable of neutralizing antibody, which neutralizes the OBM/sOBM biological activity are~~ of OBM/sOBM is useful in as a medicament,

particularly as a pharmaceutical composition, particularly pharmaceutical compositions to prevent composition for preventing and/or treating bone metabolism abnormality, or as antibodies for an antibody used in immunological diagnosis of such diseases a disease. The preparations antibody of the present invention can be prepared in a formulation, and administered orally or parenterally. A formulation comprising the antibodies of the present invention can be administered either orally or non-orally. Such preparations can be safely administered to humans or animals as a pharmaceutical composition comprising the antibody which contain the antibodies recognizing OBM and/or sOBM as an active component ingredient. As the forms Illustrative examples of the formulation of the pharmaceutical composition; injection agents including intravenous include injectable solutions such as drip, suppository agents, nasogastric agent, sublingual agents, percutaneous absorption agents, agent and transdermal agent. Since the like are given. Because monoclonal antibodies are macromolecule proteins, they not only readily adhere antibody has a high molecular weight, its adsorption to a glass containers such as a vial or a and syringe tube is significant. Furthermore, but also are the antibody is unstable and easily denatured by inactivated due to various physicochemical factors such as heat, pH, or and humidity. Therefore Thus, to stably formulate the preparations should be stabilized by the addition of stabilizers antibody, stabilizer, pH adjusters, buffering agents buffer, solubilizing agents, or detergent surfactant and the like are added thereto. As Illustrative examples of the stabilizers, stabilizer include amino acids such as glycine and alanine, saccharides such as dextran 40 and mannose, and sugar alcohols such as sorbitol, mannitol, and xylitol can be given xylitol. These stabilizers may be used either individually or in combinations of two or more. The amount of These stabilizers to be added is are preferably from added in an amount which is 0.01 to 100 times, particularly preferably from 0.1 to 10 times, as much as the amount weight of the antibody. The By addition of these stabilizers, increases the storage stability of liquid preparations formulation or lyophilized products thereof freeze-dried formulation can be improved. Phosphate buffers and citrate buffers are given as Illustrative examples of the buffering agents buffer include phosphate buffer and citrate buffer. The buffering agents not only adjust buffer adjusts the pH of the liquid preparations or an aqueous solutions obtained by reformulation or a

reconstituted solution of freeze-dissolving the lyophilized products thereof dried formulation, but also increase and thereby contributes to the stability and solubility of the antibody therein. It is desirable to add the buffering agent in an The amount to make from of the buffer is preferably, for example, 1 mM to 10 mM concentration of the liquid preparation or of the in an aqueous formulation or a reconstituted solution prepared from the lyophilized product of freeze-dried formulation. Polysorbate The surfactant is preferably polysorbate 20, Pulluronic PLURONIC® (BASF Co., Co.) F-68, 68 and polyethylene glycol are given as examples of the detergent. A, particularly preferred example is Polysorbate 80, preferably polysorbate 20. These detergents may be used either individually or in combinations of two or more. Macromolecule proteins such as A protein having high molecular weight like an antibody is easily adherable to adsorb to glass containers or resin, which a container is made of. Adherence to containers However, by addition of a surfactant adsorption of the antibody in a liquid preparation or to a container in an aqueous formulation or a reconstituted solution prepared by reof freeze-dissolving a lyophilized product dried formulation can be prevented by adding such detergents at a concentration from. The surfactant is preferably added in an amount of 0.001 to 1.0% of the weight of an aqueous formulation or a reconstituted solution of freeze-dried formulation. The preparations formulation comprising the antibodies of the present invention can be obtained prepared by adding stabilizers addition of the stabilizer, buffering agents, or agents which prevent buffer and adsorption-preventing agent as described above. When the preparations are Particularly, when it is used as injection agents an injectable formulation for medication medical applications or for treating animals, such injection agents should preferably have an acceptable osmotic pressure ratio of is preferably 1 to or 2. The osmotic pressure ratio can be adjusted by increasing or decreasing the amount of sodium chloride when making the preparations in formulation. The amount content of an the antibody in a the preparation formulation can be suitably adjusted depending appropriately, dependent on the disease to be treated with said formulation, route of administration, route and the like. A The dose of a the human-type antibody administered to humans may be changed depending depends on the affinity of the antibody to human OBM/sOBM, especially, that is, the dissociation constant (Kd value) of the antibody to human OBM/sOBM. The higher the affinity is (or the lower

~~the K_d value is), the smaller the K_d value), the less the dose that is required to be administered to humans to obtain a certain exhibit medicinal effect benefits. Because a human-type antibody has a long~~ Furthermore, since the half-life time of human-type antibodies in human blood ~~is~~ about 20 days, it is sufficient to administer ~~it~~ the human-type antibody can be administered to humans at ~~in~~ a dose of about 0.1- to 100 mg/kg at least once or more in ~~a~~ within 1- to 30 day period ~~days~~, for example.

BRIEF DESCRIPTION OF THE DRAWINGS

~~Figure~~ Fig. 1 shows the results of SDS-PAGE of mouse OBM protein of Example 3 of the present invention ~~obtained in Example 3~~, wherein:

<Explanation of symbols>

- (A): Lane 1: M molecular weight markers ~~marker~~,
Lane 2: — A partially purified sample (fraction eluted with Gly-HCl (pH 2.0) elution fraction) ~~obtained, which was derived~~ from ST2 cells cultured in the presence of the active-form of vitamin D₃ and dexamethasone,
Lane 3: — A partially purified sample (fraction eluted with Gly-HCl (pH 2.0) elution fraction) ~~obtained, which was derived~~ from ST2 cells cultured in the absence of the active-form of vitamin D₃ and dexamethasone,
(B): Lane 1: M molecular weight markers ~~marker~~,
Lane 2: M mouse OBM protein (Example 3) of the present invention after ~~purification by reverse~~ purified with reversed phase high performance liquid chromatography (Example 3).

~~Figure~~ Fig. 2 shows the results of the binding assay ~~experiment~~ of the ¹²⁵I -labeled OCIF to ~~osteoblastic~~ osteoblast-like stromal cells, ST2, in Example 4.

~~Figure~~ Fig. 3 shows the osteoclastogenesis ~~formation capability supporting~~ activity of ~~osteoblastic~~ osteoblast-like stromal cells cell line, ST22, ~~from~~ with different generations passage numbers, in Example 5(1), wherein:

<Explanation of symbols>

- 1: Ability osteoclastogenesis-supporting activity of ST2 cells ~~from about~~ with a passage number of around 10th ~~subculture to support osteoclast formation~~.

2: Ability osteoclastogenesis-supporting activity of ST2 cells from about with a passage number of around 40th subculture to support osteoclast formation's.

~~Figure Fig. 4 shows a change with the passage of time in expression of the protein of the present invention on the an osteoblast-like stromal cell membrane of osteoblastic stromal, said cells were cultured in the presence of an active-form of vitamin D₃ and dexamethasone, with passage of time, in Example 5(2).~~

~~Figure Fig. 5 shows a change with the passage of time in osteoclast formation osteoclastogenesis in the co-culture system, with passage of time, of Example 5 (2).~~

~~Figure Fig. 6 shows the inhibitory effect on osteoclast formation osteoclastogenesis-inhibiting effects when OCIF was treated with OCIF for different culture only during various culturing periods during in the co-culture period in of Example 5(3).~~

~~Figure Fig. 7 shows the results of a crosslinking test experiment of the ¹²⁵I-labeled OCIF with the protein of the present invention, in Example 6-6, wherein:~~

~~<Explanation of symbols>~~

~~Lane 1: ¹²⁵I-labeled OCIF-CDD1,~~

~~Lane 2:- sample resulting from crosslinking of ¹²⁵I-labeled OCIF-CDD1 crosslinked with ST2 cells1 with an ST2 cell line,~~

~~Lane 3:- ¹²⁵I-labeled OCIF-CDD1 crosslinked sample resulting from crosslinking an ST2 cell in the presence of a 400-fold excess higher concentration of unlabeled OCIF than that of ¹²⁵I-OCIF.~~

~~Figure Fig. 8 shows the results of SDS-PAGE in Example 9-9, wherein:~~

~~<Explanation of symbols>~~

~~Lane 1: ——— Proteins of pOBM291-transfected precipitate resulting from immuno precipitation of the protein of COS-7 cells immunoprecipitated in the absence of transfected with pOBM291 without OCIF,~~

~~Lane 2: ——— Proteins of pOBM291-transfected precipitate resulting from immuno precipitation of the protein of COS-7 cells transfected with pOBM291 with OCIF.~~

~~———— immunoprecipitated in the presence of OCIF~~

FigureFig. 9 shows the results of analysis of binding capability experiment of ^{125}I -labeled OCIF to COS-7 cells transfected with pOBM291 in Example 10.10, wherein:

<Explanation of symbols>

Lanes 1 and 2:—The amount of the ^{125}I -labeled OCIF bindingbound to COS-7 cells transfected with pOBM291291,

Lanes 3 and 4:—The amount of the ^{125}I -labeled OCIF bindingbound to COS-7 cells transfected with pOBM291 in the presence of a 400-fold excesshigher concentration of unlabeled OCIF than that of ^{125}I -OCIF.

FigureFig. 10 shows the results of a crosslinking test experiment using OCIF- ^{125}I -labeled with ^{125}I OCIF in Example 11.11, wherein:

<Explanation of symbols>

Lane 1: ^{125}I -labeled OCIF,

Lane 2: sample resulting from crosslinking of ^{125}I -labeled OCIF crosslinked with COS-7 cells transfected with pOBM291291,

Lane 3: sample resulting from crosslinking of ^{125}I -labeled OCIF crosslinked with COS-7 cells transfected with pOBM291 in the presence of a 400-fold excesshigher concentration of unlabeled OCIF than that of ^{125}I -OCIF.

FigureFig. 11 shows the results of a Northern Blot in Example 12.12, wherein:

<Explanation of symbols>

Lane 1: RNA originatingderived from ST2 cells cultured without additionin the absence of V itamin D and dexamethasone,

Lane 2: RNA originatingderived from ST2 cells cultured within the additionpresence of V itamin D and dexamethasone.

FigureFig. 12 shows the OCIF- binding capabilityability of thea proteins in the conditioned medium at variouswhen the concentration of OCIF concentrationswas varied in Example 1314-(2)-, wherein:

<Explanation of symbols>

○: pCEP44,

●: pCEP sOBM.

~~Figure~~Fig. 13 shows the OCIF- binding ~~capability~~ability of the protein in the conditioned medium at ~~various proportions~~when the amount of the conditioned medium was ~~varied~~ in Example 1314-(2)-, wherein:

<Explanation of symbols>

□: PCEP4

○: pCEP4,

●: PpCEP sOBM.

~~Figure~~Fig. 14 shows the results of SDS-PAGE of a fusion protein ~~consisting of~~ thioredoxin and mouse OBM expressed in ~~Escherichia~~E. coli, in Example 1415-(2)-, wherein:

<Explanation of symbols>

Lane 1: Mmolecular weight ~~markers~~marker,

Lane 2: Ssoluble protein fractions ~~originating~~derived from GI724/pTrxFus,

Lane 3: Ssoluble protein fractions ~~originating~~derived from GI724/pTrxOBM2525.

~~Figure~~Fig. 15 shows the OCIF- binding ~~capability~~abilities ~~at various proportions~~when the amount of the soluble protein fractions were varied in Example 1415-(3)-, wherein:

<Explanation of symbols>

□: GI724/pTrxFus,

○: GI724/pTrxOBM2525.

~~Figure~~ Fig. 16 shows the OCIF- binding ~~capability~~abilities of the soluble protein fractions (1%) ~~at various~~when the concentrations of OCIF was varied in Example 1415-(3)-, wherein:

<Explanation of symbols>

□: GI724/pTrxFus,

○: GI724/pTrxOBM2525.

~~Figure~~Fig. 17 shows the results of inhibition of the specific binding to OCIF of the mouse OBMprotein obtained by ~~expressing of~~the mouse OBM cDNA of the present invention and ~~purification or~~purifying (mouse OBM) and the purified natural-OCIF-type OCIF binding protein to OCIF, by a ~~rabbit~~an anti-mouse OBM rabbit antibody-, wherein:

<Explanation of symbols>

- 1: ~~— Purified OBM prepared by expression of the cDNA in the presence of the purified recombinant OBM treated with an antibody, OBM + 125 I-OCIF,~~
- 2: ~~The the purified natural-type protein in the presence of the treated with an antibody + 125 I-OCIF,~~
- 3: ~~— Mouse OBM prepared by expression of the cDNA in the absence of the purified recombinant OBM untreated with an antibody, mouse OBM + 125 I-OCIF,~~
- 4: ~~The the purified natural-type protein in the absence of the untreated with an antibody + 125 I-OCIF,~~
- 5: 3 + unlabeled OCIF (400-fold ~~more~~higher concentration than that of 125 I-OCIF),
- 6: 4 + unlabeled OCIF (400-fold ~~more~~higher concentration than that of 125 I-OCIF).

~~Figure~~Fig. 18 shows the results of SDS-PAGE of human OBM protein expressed by the cDNA of the present invention-, wherein:

<Explanation of symbols>

Lane 1: ~~M~~molecular weight markers~~marker,~~

Lane 2: ~~— Proteins of precipitate resulting from immuno precipitation of the protein derived from COS-7 cells transfected with phOBM (an expression vector (phOBM) containing at the cDNA of the present invention), immunoprecipitated with a rabbit by an anti-OCIF rabbit polyclonal antibody in the absence of without OCIF,~~

Lane 3: ~~— Proteins of precipitate resulting from immuno precipitation of the protein derived from COS-7 cells transfected with phOBM (an expression vector (phOBM) containing at the cDNA of the present invention), immunoprecipitated with a rabbit by an anti-OCIF rabbit polyclonal antibody in the presence of with OCIF.~~

~~Figure~~Fig. 19 shows the results of analysis of a binding experiment of OCIF to COS-7 cells transfected with phOBM, an expression vector (phOBM) containing at the cDNA of the present invention-, wherein:

<Explanation of symbols>

Lane 1: COS-7 cells transfected with phOBM ~~and the addition of~~ 125 I- OCIF,

Lane 2: COS-7 cells transfected with phOBM ~~and the addition of~~ 125 I- OCIF, ~~in the presence of~~ + a 400-fold ~~more~~higher concentration of unlabeled OCIF than that of 125 I-OCIF.

~~Figure~~Fig. 20 shows the results of crosslinking experiment of human OBM₁, which is a protein encoded by athe cDNA of the present invention; with ¹²⁵I-OCIF (monomer- type), wherein:

<Explanation of symbols>

Lane 1: ¹²⁵I-OCIF₁

Lane 2: ~~The crosslinked products sample resulting from crosslinking of~~ ¹²⁵I-OCIF with the proteins on the membrane of COS-7 cells transfected with phOBM₁;

Lane 3: ~~The crosslinked products sample resulting from crosslinking of~~ ¹²⁵I-OCIF with the proteins on the membrane of COS-7 cells transfected with ~~pHOBM₁~~phOBM₁ in the presence of a 400-fold ~~more~~higher concentration of unlabeled OCIF than that of ¹²⁵I-OCIF.

~~Figure~~Fig. 21 shows the OCIF- binding capabilityability of the protein (secretedory-formhOBMtype human OBM) in the conditioned medium at ~~various~~when the concentration of OCIF concentrations was varied in Example 2324-(2), wherein:

<Explanation of symbols>

○: ~~C~~conditioned medium of 293-EBNA cells transfected with pCEP4, ~~which does~~4 vector not containing cDNA ~~encoding secreted~~which encodes the secretory-formtype human OBM₁,

●: ~~C~~conditioned medium of 293-EBNA cells transfected with pCEPshOBM₁; expression vector containing cDNA ~~which contain~~seDNA encoding secretedencodes the secretory-formtype human OBM₁.

~~Figure~~Fig. 22 shows the OCIF- binding capabilityability of the protein (secretedory-formtype human OBM) in the conditioned medium at ~~a specific OCIF concentration~~when the amount of the conditioned medium to be added was varied while changing the amountconcentration of conditioned medium addedOCIF was kept constant, in Example 2324-(2), wherein:

<Explanation of symbols>

○: ~~C~~conditioned medium of 293-EBNA cells transfected with pCEP4, ~~which does~~4 vector not containing cDNA ~~encoding secreted~~which encodes the secretory-formtype human OBM₁,

- : Conditioned medium of 293-EBNA cells transfected with pCEPshOBM₁ expression vector containing cDNA which contains DNA encoding secreted encodes the secretory-form type human OBM₁.

Figure Fig. 23 shows the results of SDS-PAGE of a fusion protein consisting of thioredoxin and human OBM₁ expressed in Escherichia coli, wherein:

<Explanation of symbols>

Lane 1: Molecular weight markers marker,

Lane 2: Soluble protein fractions originating derived from Escherichia coli GI724/pTrxFus,

Lane 3: Soluble protein fractions originating derived from Escherichia coli GI724/pTrxhOBMpTrxOBM.

Figure Fig. 24 shows the OCIF-binding capability ability of the fusion protein consisting of thioredoxin and human OBM to OCIF, when the to bind OCIF when the amount of the soluble protein fraction originating from Escherichia containing the fused protein of thioredoxin and human OBM expressed in E. coli including the fusion protein added was varied, in Example 2425-(3), wherein:

<Explanation of symbols>

- : Soluble protein fractions originating derived from Escherichia coli GI724/pTrxFus,

- : Soluble protein fractions originating derived from Escherichia coli GI724/pTrxshOBM.

Figure Fig. 25 shows the OCIF-binding capability ability of the fusion protein of thioredoxin and human OBM in a soluble protein fractions originating from Escherichia fraction of E. coli to bind OCIF in various concentrations when the concentration of OCIF was varied, in Example 2425-(3), wherein:

<Explanation of symbols>

- : Soluble protein fractions originating derived from Escherichia coli GI724/pTrxFus

- : Soluble protein fractions originating derived from Escherichia coli GI724/pTrxshOBM.

~~Figure~~Fig. 26 shows the results of quantifying measurement of human OBM and human sOBM by the sandwich ELISA method using the rabbit anti-human OBM/sOBM rabbit polyclonal antibody of the present invention., wherein:

~~<Explanation of symbols>~~

- : Hhuman OBM₁
- : Hhuman sOBM₁

~~Figure~~Fig. 27 shows the results of quantifying measurement of human OBM and human sOBM by the sandwich ELISA method using the anti-human OBM/sOBM monoclonal antibodies of the present invention., wherein:

~~<Explanation of symbols>~~

- : Hhuman OBM₁
- : Hhuman sOBM₁

~~Figure~~Fig. 28 shows the results of quantifying measurement of mouse OBM and mouse sOBM by the sandwich ELISA method using the anti-human OBM/sOBM monoclonal antibodies of the present invention-which, said antibody has cross-reactivity to both mouse OBM and mouse sOBM., wherein:

~~<Explanation of symbols>~~

- : Mmouse OBM₁
- : Mmouse sOBM₁

~~Figure~~Fig. 29 shows the activity of the fusion protein consisting of thioredoxin and mouse OBM to stimulate promote the formation of human osteoclast-like cell formation: cells

~~Figure~~Fig. 30 shows the suppression of the vitamin D₃-stimulated bone resorption by an anti-OBM/sOBM antibody of the bone resorption activity stimulated by vitamin D₃.

~~Figure~~Fig. 31 shows the suppression of the anti-OBM/sOBM antibody of the bone resorption activity stimulated by prostaglandin E₂ (PGE₂)-stimulated bone resorption by an anti-OBM/sOBM antibody.

~~Figure~~Fig. 32 shows the suppression by the anti-OBM/sOBM antibody of the bone-resorbing activity stimulated by parathyroid hormone (PTH)-stimulated bone resorption by an anti-OBM/sOBM antibody.

~~Figure~~Fig. 33 shows the suppression by the of interleukin 1 α (IL-1)-stimulated bone resorption by an anti-OBM/sOBM antibody of the bone-resorbing activity stimulated by interleukin 1 α (IL-1).

BEST MODE FOR CARRYING OUTPRACTICING THE INVENTION

[Examples]

The present invention ~~will be described~~is explained in more detail by way of examples which are given ~~for~~with reference to the purpose of illustration ~~of~~following Examples. However, these Examples are only exemplary and shall not limit the present invention and are not limiting thereof ~~thereto~~ in any way of the remainder of the disclosure.

<[Example 1]>

Preparaoduction of the protein of the present invention

(1) Large-~~s~~Scale-cultivation Culture of ST2 ~~e~~Cells

Mouse osteoblasticosteoblast like stromal cell line, ST2~~2~~, (~~RIKEN CELL~~ BANKRiken Cell Bank, RCB0224) was cultured ~~using~~with α -MEM medium containing 10% fetal-bovine fetal serum. ST2-cells~~After~~ cultured to ~~e~~confluencebecome confluent in a 225 -cm² T flask for adherent-cell-culture cells, ST2 cells were treated with trypsin and harvested, stripped from the T flask. ~~After washing, the cells were~~washed, and then transferred to five of 225 -cm² T flasks. After the addition of 60 ml of α -MEM medium containing 10⁻⁸ M of the active-form of vitamin D₃ (Calcitriol), 10⁻⁷ M dexamethasone, and 10%bovine fetal bovine-serum, the resulting cells in each flask were cultured for 7-10 days in a CO₂ incubator for 7 to 10 days. The cultured ST2 cells were harvestedrecovered using a cell scraper and stored at -80°C until use.

(2) Preparation of ~~m~~Membrane ~~f~~Fraction and ~~s~~Solubilization of ~~m~~Membrane-bound ~~p~~Proteins

To the ST2 cells (volume,amount: about 12 ml) described in Example 1-(1), which were cultured ~~using eighty~~with 80 of 225 -cm² T flasks, ~~was added three times~~

thea 3-fold volume (36 ml) of 10 mM Tris-HCl hydrochloric acid buffer (pH 7.2) containing protease inhibitors (2 mM APMSFP, 2 mM EDTA, 2 mM o-phenanthroline, 1 mM leupeptin, 1 μ g/ml pepstatin A; and 100 units/ml aprotinin) were added. After these cells were vigorously agitatingagitated by use of a vortex mixer for 30 seconds using a voltex mixer, the cellsthey were allowedleft to stand on ice for 10 minutes on ice. The cells were homogenized usingUsing a homogenizer (DOUNCE TISSUE GRINDERDounce Tissue Grinder, A syringe, WHEATONWheaton SCIENTIFICScientific Co., Ltd.), these cells were crushed. The sameTo the crushed cell solution, an equal volume (48 ml) of 10 mM Tris-HCl hydrochloric acid buffer (pH 7.2) containing the above-mentioned protease inhibitors, 0.5 M sucrose, 0.1 M potassium chloride, 10 mM magnesium chloride; and 2 mM calcium chloride was added to the homogenized cells. After stirring, theThe obtained mixture was agitated and then centrifuged at 600 x g at 4°C for 10 minutes at 4°C. Through this centrifugation, thereby separatingcell nuclei and non-homogenizeduncrushed cells were separated as precipitateprecipitated fractions. TheA supernatant obtained by the centrifugeafter centrifugation was further centrifuged at 150,000 x g at 4°C for 90 minutes at 4°C, to obtainand membrane fractions of the ST2 cells were obtained as precipitateprecipitated fractions. EightTo the membrane fractions, 8 ml of 10 mM Tris-HCl hydrochloric acid buffer (pH 7.2) containing the above-mentioned protease inhibitors, 150 mM of sodium chloride, and 0.1 M sucrose was added to this membrane fraction. After the addition of, and then 200 μ l of 20% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-dimethylammonio]-1-propanesulfonate, Sigmaaigma Co.), theLtd.) was added. The mixture was stirredagitated at 4°C for 2 hours at 4°C. Theis mixtureresolution was then centrifuged at 150,000 x g at 4°C for 60 minutes at 4°C, to obtainand the resulting supernatant was obtained as a solubilized membrane fraction.

<[Example 2]>

Purification of the pProtein of the pPresent iInvention

(1) Preparation of OCIF-iImmobilized aAffinity eColumn

After replacing iso-propanolIsopropanol in a HiTrapHITRAP® NHS-activated column (1 ml, manufactured by Pharmacia Co., Ltd.) was substituted with 1 mM hydrochloric acid, and 1 ml of 0.2 M NaHCO₃/0.5 M NaCl (pH 8.3) solution (pH 8.3)

containing 13.0 mg of recombinant OCIF prepared ~~by the~~ in accordance with a method ~~of described in~~ WO 96/26217 was added to the column using a syringe (5 ml, ~~manufactured by Terumo Corp. Corporation~~), After the column was allowed to ~~effect~~ undergo a coupling reaction at room temperature for 30 minutes. ~~The column was~~ fed with, 3 ml of 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 3 ml of 0.1 M acetic acid/0.5 M NaCl (pH 4.0) were loaded on the column alternately three times each in ~~turn~~ total so as to inactivate excessive activated groups. Then, then the solution mobile ~~phase of the column was replaced~~ substituted again with 0.5 M ethanolamine/0.5 M NaCl (pH 8.3). ~~After allowing and then left to stand at room temperature for 1 hour.~~ Thereafter, the resulting column was washed twice alternately with 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH 4.0), and and ~~then the solution mobile phase was then replaced~~ substituted with 50 mM Tris/1 M NaCl/0.1% CAHPS buffer (pH 7.5).

(2) Purification of the pProtein of the present invention using Present Invention by an OCIF-immobilized aAffinity eColumn

~~The purification~~ Purification of the OCIF- binding protein was carried out at 4°C, unless otherwise ~~indicated~~ stated. The above-mentioned OCIF-immobilized affinity column was equilibrated with 10 mM Tris-~~hydrochloride~~ hydrochloric acid buffer (pH 7.2) ~~to which~~ containing the protease inhibitors described in Example 1-(2), 0.15 M sodium chloride, and 0.5% CHAPS-~~were added~~. ~~About~~ To this column, about 8 ml of the solubilized membrane fraction described in Example 1-(2) was applied ~~to the column~~ at a flow rate of 0.01 ml/minute. ~~Then, the~~ The column was washed with the above 10 mM Tris-~~hydrochloride~~ hydrochloric acid buffer (pH 7.2) ~~to which~~ containing the above- mentioned protease inhibitors, 0.15 M sodium chloride, and 0.5% CHAPS ~~was added, for 100 minutes~~ at a flow rate of 0.5 ml/minute ~~in for 100 minutes~~. ~~Next~~ Then, the proteins ~~adsorbed to~~ were eluted from the column ~~was eluted~~ with 0.1 M glycine-~~hydrochloride~~ hydrochloric acid buffer (pH 3.3) containing the above protease inhibitors, 0.2 M sodium chloride, and 0.5% CHAPS ~~for 50 minutes~~ at a flow rate of 0.1 ml/minute ~~in for 50 minutes~~. ~~In the same manner~~ Similarly, the proteins adsorbed to the ~~column was eluted with~~ a 0.1 M sodium citrate buffer (pH 2.0) containing the said protease inhibitors, 0.2 M sodium chloride, and 0.5% CHAPS ~~for 50 minutes~~ was fed to

the column at a flow rate of 0.1 ml/minute for 50 minutes so as to elute proteins
adsorbed to the column. ~~The eluates was collected in~~ were fractionated as 0.5 ml/fraction
each. ~~The fractions. Each fraction was~~ were immediately neutralized by the addition of a
2M Tris solution. ~~The fractions derived from the elution with these buffers (each fraction~~
~~consisting the volume of the eluate was 1.0- to 5.0 ml of eluate)~~ eluted with the buffer
were concentrated to 50- to 100 μ l using Centricon 10 (manufactured by Amicon of
UCENTRICON®-10 (Amersham Co.S.A, Ltd.). ~~OCIF was added to a portion of~~
~~each~~ Aliquots of the concentrated fraction ~~fractions were subfractionated, and after~~
addition of OCIF to the aliquots, they were immunoprecipitated with an anti-OCIF
polyclonal antibody. ~~The~~ After the precipitated fractions were treated with SDS and, they
were subjected to SDS-PAGE. ~~Fractions, and then a fraction (Fr. No. Nos. 3-10) in~~
~~which the~~ showing a band of the protein with specific binding ability having an activity to
specifically bind OCIF appeared were regarded as identified as the protein fractions of
the present invention.

(3) Purification of the pProtein of the pPresent iInvention by gGel fFiltration

The concentrated-OCIF- binding protein (the fractions obtain by the elution eluted
with 0.1 M glycine-hydrochloride/hydrochloric acid buffer (pH 3.3) and subsequently 0.1
M sodium citrate buffer (pH 2.0)) ~~prepared after purification and concentration in~~
accordance with the method described in Example 2-(2) was applied ~~subjected to a~~
Superose ~~SUPEROSE® 12 HR10/30 column (1.0 x 30 cm, manufactured by Pharmacia~~
Co., Ltd., 1.0 X 30 cm) ~~which was equilibrated with 10 mM Tris-HCl, 0.5 M NaCl, and~~
0.5% CHAPS (pH 7.0) and developed with using the above equilibration buffer as a
mobile phase at a flow rate of 0.5 ml/min, and each then fractions of 0.5 ml fraction
was/were collected. The fractions containing the protein of the present invention (Fr. Nos.
Nos. 27-32) were/was identified according to and concentrated by means of
CENTRICON®-10 (Amersham Co., Ltd.) in the same method/manner as described
above. ~~Each of the fractions was concentrated using Centricon 10 (a product of~~
Amicon).

(4) Purification by rReversed pPhase hHigh pPerformance lLiquid
eChromatography

~~The above mentioned~~ OCIF- binding protein purified by the above gel filtration was ~~applied~~added to a C₄ column (2.1 ~~x~~X 250 mm, Vydac, USA) ~~which was equilibrated~~ with 0.1% trifluoroacetic acid (TFA) and 30% acetonitrile. ~~The proteins bound to the column were eluted~~Elution was carried out at a flow rate of 0.2 ml/min with linearthe gradients of acetonitrile concentration of from 30% to 55% for the first 50 minutes and then of from 55% to 80% during the next for another 10 minutes at a flow rate of 0.2 ml/min. ~~Peaks~~The peaks of eluted proteins were detected by measuring optical density at 215 nm~~mm~~. ~~Proteins in the different peaks were analyzed to identify the fractions containing~~The eluted protein of each peak was fractionated, and the peak of the protein of the present invention, and was identified. Thus, a highly purified protein of the present invention was obtained.

<[Example 3]>

SDS-PAGE of the pPurified pProtein of the pPresent iInvention

~~The~~First, a solubilized membrane fraction prepared from ST2 cells which were cultured in the presence or absence of the active-form of vitamin D₃ was ~~subjected to purification~~purified with the OCIF-immobilized affinity column. ~~The as described above, and the purified preparations~~samples were subjected to SDS-PAGE. As shown in ~~Figure~~Fig. 1(A), it was revealed that a major protein band with MW of about 30,000- to 40,000 was detected only in the purified ~~preparations~~sample obtained from the ST2 cells ~~which was cultured in the presence of the active-form of~~ vitamin D₃, ~~proving and that the~~ protein which specifically binds to OCIF, (i.e., the protein of the present invention) ~~can be,~~ is selectively concentrated and purified bywith the OCIF-immobilized affinity column. However, ~~bands of several proteins (other than~~in addition to the protein of the present invention), ~~some other bands of proteins which non-specifically were nonspecifically bound to the carriers or, spacers or the like of the OCIF-immobilized column were also detected in both of the purified preparations~~samples. These proteins other than the protein of the present invention were removed ~~according to the above-described method by~~ gel filtration and C₄ reversed phase chromatography as described above. The SDS-PAGE of the obtained highly purified protein of the present invention is

shown in Figure Fig. 1(B). The highly purified protein of the present invention was found to be electrophoretically homogeneous, and had ~~at the~~ molecular weight ~~of thereof~~ was about 30,000- to 40,000.

<[Example 4]>

Examining the Binding test of OCIF to Osteoblasts

(1) Preparation of ^{125}I -Labeled OCIF

OCIF was ~~^{125}I -labeled with ^{125}I~~ by the Iodogen method. ~~Specifically~~ More specifically, 20 μl of 2.5 mg/ml Iodogen-chloroform solution was transferred to a 1.5 ml Eppendorf tube, and chloroform was evaporated off at 40°C , ~~to obtain a tube coated with~~ so as to prepare an Iodogen-coated tube. ~~The~~ After the tube was washed three times with 400 μl of 0.5 M sodium phosphate buffer (Na-Pi, pH 7.0). ~~Five μl , 5 μl~~ of 0.5 M Na-Pi (pH 7.0) was added ~~to the tube thereto~~. Immediately after the ~~addition of 1.3 μl~~ (18.5 MBq) of Na- ^{125}I solution (NEZ-033H20, ~~manufactured by Amersham Co.~~), Ltd., NEZ-033H20) was added to the tube, 10 μl of 1 mg/ml rOCIF solution (monomer type or dimer type) was added ~~to the tube~~. ~~After mixing~~ The obtained solution was agitated with a vortex mixer, ~~the mixture was allowed and then left~~ to stand at room temperature for 30 seconds. The solution was transferred to a tube containing 80 μl of a solution of 10 mg/ml potassium iodide ~~in and~~ 0.5 M Na-Pi solution (pH 7.0), and 5 μl of a phosphate buffered saline solution containing 5% bovine serum albumin, and ~~stirred then agitated~~. The mixture solution was applied to a spin column (1 ml, G-25 fine, ~~manufactured by Pharmacia Co., Ltd.~~) which was equilibrated with a phosphate buffered saline solution containing 0.25% bovine serum albumin and ~~the column was~~ centrifuged at 2,000 rpm for 5 minutes ~~at 2,000 rpm~~. ~~Four hundred μl~~ After adding 400 μl of a phosphate buffered saline solution containing 0.25% bovine serum albumin ~~was added to the fraction eluted from the column and the mixture was stirred~~. ~~A subsequently mixed, 2 μl of the aliquot was removed to measure~~ aliquots were collected, and the radioactivity using thereof was measured with a gamma counter. The radiochemical purity of the thus prepared ^{125}I -labeled OCIF solution was determined by measuring the radioactivity of a fraction precipitated ~~with~~ by 10% TCA. ~~The~~ Furthermore, the biological activity of as OCIF the ^{125}I -labeled OCIF solution was ~~measured according to the~~ determined in accordance with a method described in WO 96/26217. ~~The~~ Moreover, the concentration

of the ^{125}I -labeled OCIF was measured by the ELISA according to the following procedure manner.

(2) Measurement of the concentration of ^{125}I -Labeled OCIF by ELISA

~~One hundred~~ 100 μl of 50 mM NaHCO_3 (pH 9.6) in which ~~the~~ 2 $\mu\text{g/ml}$ of anti-OCIF rabbit polyclonal antibody described in WO 96/26217 was dissolved to a concentration of 2 $\mu\text{g/ml}$ was added to each well of a 96-well immunoplate (MaxiSorpTM, a product of Nunc Co.). ~~The plate was allowed, Ltd.) and left to stand at 4°C overnight at 4°C.~~ After removing the this solution by ~~was~~ suction removed, 300 μl of Block-AceTM BLOCKACE (Snow Brand Milk Products Co., Ltd.)/phosphate buffered saline (25/75)-solution (25/75) was added to each well. ~~The plate was and then allowed left to stand for two hours at room temperature. for 2 hours.~~ After removing the this solution by suction, the ~~was removed, each wells were was~~ washed three times with phosphate buffered saline solution (P-PBS) containing 0.01% Polysorbate 80 (P-PBS).polysorbate 80. Next ~~Thereafter~~, 300 μl of Block-AceTM BLOCKACE/phosphate buffered saline (25/75)-solution to which (25/75) containing ^{125}I -labeled OCIF sample or the standard OCIF preparation was mixed, was added to each well. ~~The plate was then allowed and left to stand for two hours at room temperature for 2 hours.~~ After removing the this solution by ~~was~~ suction removed, each well was washed six times with 200 μl of P-PBS. ~~One hundred μl~~ Then, 100 μl of Block-AceTM BLOCKACE (Snow Brand Milk Products Co., Ltd.)/phosphate buffered saline (25/75)-solution (25/75) containing peroxidase -labeled rabbit anti-OCIF rabbit polyclonal antibody was added to each well. ~~The plate was allowed and left to stand for two hours at room temperature for 2 hours.~~ After removing the this solution by ~~was~~ suction removed, the ~~each wells were was~~ washed six times with 200 μl of P-PBS. ~~Then, 100 μl~~ Then, 100 μl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well. ~~After incubating and then left to stand at room temperature for 2- to 3 minutes. Thereafter, 100 μl of stopping solution (Stopping Reagent, (Scytek Co., Ltd.) was added to each well.~~ Absorbance ~~The absorbance~~ of each well at 490 nm was measured at 490 nm using with a microplate reader. The concentration of the ^{125}I -labeled OCIF was ~~determined~~ calculated from a calibration curve ~~prepared made by using the standard preparation of OCIF.~~

(3) Examining the Binding test of OCIF to Osteoblasts or spleenPancreas eCells

Mouse ~~osteoblastic~~osteoblast-like stromal cell line, ST22, or ~~spleen~~mouse pancreas cells were suspended in α -MEM medium containing 10% bovine fetal bovine serum (FBS), either with or without 10^{-8} M of the active-form of vitamin D₃ (Calcitriol) and 10^{-7} M dexamethasone, to at a concentration of 4×10^4 cells/ml (ST22 cells) or 2×10^6 cells/ml (spleen cells), respectively. Each cell suspension 1 ml of this medium was inoculated seeded into a 24- well micro-platemicroplate. TheAfter the cells were cultured for 4 days in in a CO₂ incubator. After washing the cells for 4 days and washed with α -MEM medium, 200 μ l of medium for the binding testexperiment (α -MEM to whichmedium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, and 0.2% NaN₃ were supplemented); further containing 20 ng/ml of the above-described ¹²⁵I-labeled OCIF (monomer formtype or dimer formtype); was added to each well. To the wells for the measurement of non-specific bindingFurthermore, 200 μ g/ml of the medium for the binding testexperiment containing 8 μ g/ml of rOCIF (400-times-fold higher concentration) in addition to 20 ng/ml of ¹²⁵I-labeled OCIF was added. The to other wells which were subjected to measurements of nonspecific binding. After the cells were cultured for one hour in a CO₂ incubator andfor 1 hour, they were washed 3three times with 1 ml of a-phosphate buffered saline solution. In this procedureSince pancreas cells are floating cells, spleen-cells in each well were washed by centrifugingin the 24-well plate in each washing step, because the spleen-cells were non-adherentwith centrifugation. After washing, 500 μ l of 0.1 N NaOH solution was added to each well and the plate was allowedleft to stand for 10 minutes at room temperature to dissolvefor 10 minutes. Thereby, the cells. The were washed, and the amount of RI in each wellbound to the cells was measured bywith a gamma counter.

As shown in Figure 2, The ¹²⁵I-labeled OCIF did not bind to the cultured spleenpancreas cells, but specifically bound only to the osteoblasticosteoblast like stromal cells which were cultured in the presence of the active-form of vitamin D₃. The results indicatedThereby, it was revealed that the protein of the present invention iswas a membrane -bound protein induced byon the cell surface of osteoblast like stromal cells with the active-form of vitamin D₃ and dexamethasone on osteoblastic stromal cells.

<[Example 5]>

Biological aActivity of the pProtein of the pPresent iInvention

(1) — ~~Osteoclasts formation supported by osteoblastic stromal cells~~

(1) Ability of Osteoblast Like Stromal Cells to Support Osteoclast Formation

~~The osteoclasts ability of osteoblasts to support osteoclast formation supporting capability of osteoblastic stromal cells was evaluated~~examined by measuring tartaric acid resistant acid phosphatase activity (TRAP activity) of the formed osteoclasts.

~~Specifically~~More specifically, spleen mouse osteoblast-like stromal cell line, ST2, (5 X 10³ cells/100 μ l/well) (2 ~~x~~ X 10⁵ cells/100 μ l/well) and pancreas cells derived from a ddy mouse (8- to 12 weeks old) and mouse osteoblastic stromal cells ST2 (5 x 10³ cells/100 μ l/well) were suspended in α -MEM to which 10⁻⁸-medium containing 10% bovine fetal serum, 10⁻⁸ M of the active-form vitamin D₃, 10⁻⁸ of vitamin D₃ and 10⁻⁷ M dexamethasone, and 10% fetal bovine serum were addedseeded in a 96-well plate. After the cells were inoculated into 96-well plates and cultured in a CO₂ incubator for one week in a CO₂ incubator. After washing, each well was washed with phosphate buffered saline solution. Then, 100 μ l of ethanol/acetone (1:1) was further added to each the well, wells and the cells were immobilized fixed at room temperature for one minute. After immobilization Then, 100 μ l of 50 mM citratecitric acid buffer (pH 4.5) containing 5.5 mM p-nitrophenol phosphate and 10 mM sodium tartarate was added to each well. After and then allowed to react at room temperature for 15 minutes. After of the reaction at room temperature, 0.1 N NaOH solution was added to each well, and the absorbance at 405 nm was measured using with a microplate reader. The Fig. 3 shows the results of osteoclasts formation by examining the abilities of ST2 cells with a to support osteoclast formation, wherein the passage number of about said cells were around 10's or around 40's (after purchasing the cells purchased from RIKEN CELL BANK are shown in Figure 3-Riken Cell Bank). The From these results indicate, it was revealed that the ST2 cells with of a higher passage number exhibit more potent had a high ability to support osteoclasts- formation.

(2) — ~~Time course change of expression of the protein of the present invention on~~

~~membrane of osteoblastic stromal cells in a culture system which include active-form vitamin D₃ and dexamethasone and time course change~~

(2) Changes with the Passage of Time in Expression of osteoclasts-formation the Protein of the Present Invention on the Membrane of Osteoblast Like Stromal Cells Cultured in the co-culture system the Presence of the Active-Form of Vitamin D₃ and Dexamethasone and Those in Osteoclast Formation in a Co-Culture System

~~In the same manner as in Example 4(3), osteoblastic~~ Osteoblast-like stromal cell line, ST2₂, was cultured ~~for 7 days~~ in the presence of the active-form of vitamin D₃ and dexamethasone for 7 days in the same manner as in Example 4-(3). ~~The OCIF-binding test experiment was carried out~~ conducted using ¹²⁵I-labeled OCIF (monomer type) as described in ~~the experiment in~~ Example 4-(1). ~~Non-specific~~ Nonspecific binding was measured by competing the ¹²⁵I-labeled OCIF binding to ST2 cells with a 400-fold higher concentration of unlabeled OCIF in binding to ST2 cells. As a result, ~~it was confirmed that the amount of specific binding of the~~ ¹²⁵I-labeled OCIF increase in accordance with increase in culture period in was increased, due to the presence of active-form of vitamin D₃ and dexamethasone, with an increase in culturing days. ~~Specifically~~ That is, as shown in Figures 4 and 5, the protein of the present invention was expressed on the cell surface of ST2 cells ~~by~~ due to the active-form of vitamin D₃ in accordance with an increase in culture period days, and ~~the~~ its expression reached a maximum on the fourth day of culture. On the other hand, osteoclast-like cells ~~are~~ were formed ~~by co-culturing after co-culture of~~ mouse spleen cells and ST2 cells in the presence of the active-form of vitamin D₃. TRAP (a marker enzyme ~~of~~ for osteoclasts) ~~—~~ positive mononuclear pre-osteoclast-like cells are were formed on the third or fourth day of ~~the~~ culture. ~~More, and further,~~ differentiated and matured TRAP-positive multinuclear cells are were formed on the fifth ~~to~~ or sixth day of ~~the~~ culture. ~~A good correlation between~~ It was found that change with the passage of time—course of the in expression of the protein of the present invention and osteoclasts-in osteoclast formation was thus demonstrated corresponded well with each other.

(3) ~~Inhibition of osteoclasts formation by OCIF treatment for different period during the co-culture~~

(3) Effect of Inhibiting Osteoclast Formation When OCIF Was Treated Only During a Restricted Period of Co-Culture

To make it clear ~~further clarify~~ that the protein of the present invention ~~is~~ was a factor involved in ~~the osteoclast osteoclasts~~ formation, the cells cultured during various periods (two days each, except for the fifth day) were treated with 100 ~~mgng/ml~~ ng/ml of OCIF ~~for different culture periods during the six in the above 6-day co-culture period described in the above mentioned Example 5(2) (two consecutive days in the six day period, except for the 5th day for which a one-day period was applied(2)).~~ As a result, ~~as shown in Figure~~ Fig. 6, in the case where OCIF treatment at was added during the 48-th to 96-hours after start of ~~th hr (as counted from the beginning of culture at which expression of), when~~ the protein of the present invention was expressed at highest level on ST2 cells ~~is~~ maximal ~~was found to be most effective for inhibiting, osteoclast formation of~~ osteoclasts was inhibited most effectively. Specifically That is, it was confirmed revealed that OCIF ~~controls~~ inhibited osteoclast formation by binding to ST2 cells via the protein of the present invention.

~~Based on the results of~~ From the above experiments results, it became clear that the protein of the present invention ~~was confirmed to be induced on cell membrane of osteoblast~~ the membranes of osteoblast-like stromal cells by ~~with the active-form of~~ vitamin D₃ and dexamethasone, and to exhibit ~~had the biological activity to support or accelerate(effect) of a factor which supports and promotes differentiation or and~~ maturation of osteoclasts.

<[Example 6]>

Crosslinking test for Experiment of ¹²⁵I-labeled OCIF and to the p Present i Invention

To further identify the presence of the protein of the present invention ~~more~~ clearly, ¹²⁵I-labeled OCIF was allowed to crosslink with the protein of the present invention ~~was crosslinked with~~ ¹²⁵I-labeled OCIF. ~~Mouse osteoblastic stromal~~ As in Example 4-(3), mouse osteoblast like cell line, ST22, was cultured for four days in the presence or absence of the active-form of vitamin D₃ and dexamethasone in the same manner as described in Example for 4(3) days. After ~~washing the cells were washed with~~ 1 ml of phosphate buffered saline solution, 200 µl of medium for binding

test experiment (α -MEM to which medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.2% NaN_3 and 100 $\mu\text{g/ml}$ heparin were added), further containing 25 ng/ml of the above ^{125}I -labeled OCIF (monomer type) or 40 ng/ml of ^{125}I -labeled OCIF-CDD1 which was added. ^{125}I -labeled OCIF-CDD1 was obtained by expressing the protein of Sequence described as SEQ ID No. NO: 76 (in WO 96/26217) in with animal cells, was added. The and labeling in accordance with the above-mentioned culture method. Furthermore, the medium for the binding test was further supplemented with experiment, containing a 400-fold higher concentration of OCIF and, was added to the other well well and was subjected to assess non-specific experiment for nonspecific binding. After culturing for one hour the cells were cultured in a CO_2 incubator for 1 hour, each well was they were washed three times with 1 ml of phosphate buffered saline solution containing 100 $\mu\text{g/ml}$ of heparin. Five Then, hundred 500 μl of phosphate buffered saline containing solution in which 100 $\mu\text{g/ml}$ of crosslinking agent, DSS (Disuccinimidyl suberate, Pierce Co.), Ltd.) was dissolved was added to each well thereto, and the plate was kept allowed to react at 0°C for 10 minutes at 0°C . The After the cells in these wells were washed twice with 1 ml of phosphate buffered saline at solution cooled to 0°C . One, hundred 100 μl of 20 mM Hepes buffer containing 1% Triton X-100, 10 μM pepstatin, 10 μM leupeptin, 2 mM PMSF (phenylmethylsulfonyl fluoride), 10 μM pepstatin, 10 μM leupeptin, 10 μM antipain, and 2 mM EDTA, was then added to each well. The plate was allowed, and left to stand for 30 minutes at room temperature to dissolve for 30 minute so as to lyse the cells. Fifteen After 15 μl of these samples were treated with SDS under non-reducing conditions according to conventional method and subjected to in accordance with a commonly used method, they were run on a SDS-polyacrylamide gel electrophoresis (4-gel (with a gradient of 4 to 20% polyacrylamide gradient, manufactured by Daiichi Chemical Pure Chemicals Co., Ltd.). After electrophoresis, the gels were was dried and exposed to BioMax BIOMAX® MS film (manufactured by Kodak Co., Ltd.) for 24 hours at -80°C using BioMax BIOMAX® MS intensifying screens (manufactured by amplifying screen (Kodak) Co., Ltd.) at -80°C for 24 hours. After exposure, the film was The exposed films were developed by in conventional accordance with a commonly used method. A band of crosslinking product with When the ^{125}I -labeled OCIF (monomer

type, 60 kDa) was used, a crosslinked protein having a molecular weight of about 90,000- to 110,000 was detected. On the other hand, when the ^{125}I -labeled OCIF (monomer type, 60 kDa) was used. When the ^{125}I -labeled OCIF-CDD1 (31 kDa) was used, a band of crosslinking product crosslinked protein of about 70- to 80 kDa (78 kDa on average, 78 kDa) was detected as shown in Figure Fig. 7.

<[Example 7]>

Scatchard Plot Analysis of the pProtein of the pPresent_ iInvention_ eExpressed on ST-cells by Scatchard Plot2 Cells

The above-mentioned ^{125}I -labeled OCIF (monomer type) was added to a concentration of 1,000 pM to the culture medium Medium for the binding test experiment (α -MEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, and 0.2% NaN_3) and the culture medium further containing 1,000 pM of the above ^{125}I -labeled OCIF (monomer type) was serially prepared and diluted stepwise at a dilution rate to of 1/2 with the culture medium not containing ^{125}I -labeled OCIF for the binding experiment. Solutions Furthermore, another medium for measuring determining non-specific non-specific binding were was prepared by further adding a 400-fold higher concentration of unlabeled monomer-form type OCIF to these solutions the above medium. Two hundred μ 200 μl of these prepared solutions were added to the above-mentioned wells with of the above ST2 cells (about 10th passage), cultured for 4 days (passage number, about 10) in the presence of 10^{-8} M of the active-form of vitamin D₃ (1,25-(OH)₂D₃) and 10^{-7} M dexamethasone, to assess and binding of the ^{125}I -labeled OCIF was tested in the same method manner as described in Example 4-(3). The obtained results were subjected to Scatchard Plot analysis to determine the plotted in accordance with a common method, and dissociation constants of OCIF and the OCIF- binding protein, and the number (site) of OCIF- of the OCIF binding protein (site) per a one ST2 cell were determined. As a result, the dissociation constants of OCIF and the protein of the present invention was found to be were 280 pM, and the number of the site of OCIF- binding protein (site) per a one ST2 cell was approximately about 33,000/cell. Based on the finding in Example 5(1) that osteoclasts formation supported by the Furthermore, a cultured ST2 cells with a passage number about number of around 40 was more extensive 's had higher ability to support osteoclast formation than that with a passage number

about 10, the number (the site) of around 10's as shown in Example 5-(1), so that the number of sites of the protein of the present invention expressed on the ST2 cell with a passage number about of around 40's was assessed measured. The As a result, the number (of site) was 58,000/cell and which, was clearly larger greater than that on the ST2 cells with a passage number about 10, indicating of around 10's. It was revealed that the amount of the expression of the protein of the present invention expressed on associated with the degree of the ability of ST2 cells is related to their potency cell to support osteoclasts- formation. The is results- finding indicateds that the protein of the present invention is a factor that to supports or induces and promote differentiation or and maturation of osteoclasts.

<[Example 8]>

Cloning of OBM cDNA

(1) Extraction of RNA from mMouse ST2 eCells

Mouse ~~osteoblastic~~ osteoblast-like stromal cell line, ST2, (~~RIKEN CELL~~ BANK Riken Cell Bank, RCB0224) was cultured in ~~α~~ with α -MEM medium (Gibco BRL Co., Ltd.) containing 10% fetal-bovine fetal serum. ST2 cells After cultured to cells become confluent in a 225 -cm² T- flasks for adherent cell culture, ST2 cells were treated with trypsin to harvest the cells, stripped from the T- flask. The cells were, washed, and transferred to five 225 -cm² T- flasks. Sixty After adding 60 ml of α -MEM medium containing 10⁻⁸ M of the active-form of vitamin D₃ (Calcitriol, Wako Pure Chemicals Co. Industries, Ltd.), 10⁻⁷ M dexamethasone, and 10% fetal-bovine fetal serum was added to each flask and thereto, the cells were cultured for 5 days in a CO₂ incubator for 5 days. Total RNA was extracted from the cultured ST2 cells using ISOGEN (Wako Pure Chemicals Co. Industries, Ltd.). Poly A⁺ RNA was prepared from about 600 μ g of the total RNA using an Oligo (dT)-cellulose column (5'-3' Prime Co., Ltd.). About 8 μ g of Ppoly A⁺ RNA was obtained.

(2) Construction of eExpression Library

Double- stranded cDNAs was were synthesized from 2 μ g of polyA the poly A⁺ RNA obtained in Example 8-(1) using a with Great Lengths cDNA Synthesis kit (Clontech Co., Ltd.) according to the instruction in the accordance with a manual thereof. Specifically More specifically, 2 μ g of polyA the poly A⁺ RNA and an Oligo (dT) 25 (dN)

primer were mixed ~~and together~~, distilled water was added ~~to the mixture to make~~ thereto so that the final volume ~~to was~~ 6.25 μ l. ~~After incubation, and the mixture was incubated~~ at 70°C for about 3 minutes at 70°C, the mixture was and then cooled ~~on~~ in ice for 2 minutes. ~~To the mixture were added~~ Then, 2.2 μ l of distilled water, 2.5 μ l of 5X First-strand buffer, 0.25 μ l of 100 mM DTT (dithiothreitol), 0.5 μ l of PRIME RNase inhibitor (1 U/ml) (5'-3' Prime Co., Ltd.), 0.5 μ l of [α -³²P] dCTP (Amersham Co., Ltd., 3,000 Ci/mmol), 2 μ Ci/ μ l) which was diluted 5-fold with distilled water to make 2 μ Ci/ μ l to be one fifth concentration, 0.65 μ l of dNTP (20 mM each), and 1.25 μ l (250 units) of MMLV (RNaseH) reverse transcriptase were added thereto, respectively. ~~The mixture~~ Thus obtained solution was incubated at 42°C for 90 minutes at 42°C. Then, followed by the further addition of 62.25 μ l of distilled water, 20 μ l of 5X second-strand buffer, 0.75 μ l of dNTP (20 mM each), and 5 μ l of Second-strand enzyme cocktail. The resulting mixture were added thereto, respectively. Thus obtained solution was maintained incubated at 16°C for two 2 hours. Then, 7.5 units of T4 DNA polymerase was added to this reaction mixture. After incubation thereto, and further incubated at 16°C for another 30 minutes. Thereafter, the reaction was terminated by the addition of 5 μ l of 0.2 M EDTA. After was added to terminate the reaction, and after a phenol-chloroform treatment, the product was precipitated with ethanol precipitation was carried out. An EcoRI-SalI-NotI linker (Clontech Co., Ltd.) was attached added to the ends of the resultant double-stranded cDNA. Then, the ends were phosphorylated and the product was applied on then phosphorylated at its end. Using a column for size fractionation column to obtain cDNA with a length more, cDNAs of not smaller than 500 bp. DNA was were separated, and ethanol-precipitated. The precipitated with ethanol, dissolved DNAs were reconstituted in water and ligated inserted to into pcDL-SR α -296 (Molecular and Cellular Biology, Vol. 8, pp. 466- to 472, 1988) which had been out (Takara Shuzo Co., Ltd.) previously cleaved with a restriction enzyme, EcoRI (Takara Shuzo Co.), and subsequently treated with CIAP (calf intestine alkaline phosphatase, Takara Shuzo Co., Ltd.).

(3) Screening of Expression Library by means of in Which the Binding to OCIF Was Used as an Index

An *Escherichia coli* strain, XL2 Blue MRF' (Toyobo Co., Ltd.), was transformed using with the DNA produced obtained in Example 8-(2), and cultured allowed to grow on a L-Carbenisilin agar Carbenicillin Agar Medium (1% trypton, 0.5% yeast extract, 1% NaCl, 60 µg/ml carbenisilin, carbenicillin and 1.5% agar) prepared in a 24-well plastic plates, plate for cell culture so that the cells was grown to produce about 100 colonies per well. Transformants The transformants in each well were suspended in 3 ml of Terrific Broth ampicillin culture-medium (1.2% trypton, 2.4% yeast extract, 0.4% glycerol, 0.017 M KH₂PO₄, 0.072 M K₂HPO₄, 100 µg/ml ampicillin), and cultured with shaking at 37°C overnight with shaking. Cells were The *E. coli* was collected by centrifugation to prepare, and plasmid DNA using a QIAwell DNAs were prepared therefrom with QIAWELL® kit (QIAGEN Co., Ltd.). The DNA concentration content was determined by measuring detecting absorbance at 260 nm. DNA, and the DNAs was concentrated by precipitating with ethanol precipitation and dissolved in distilled water to so that the concentration of was 200 ng/µl. Five hundred Thus, 500 DNA pools, each of which was obtained derived from about 100 colonies were prepared and were used for transfection into of COS-7 cells (RIKEN CELL BANK Riken Cell Bank, RCB0539). COS- 7 cells were seeded into DMEM containing 10% fetal bovine serum in each well of a 24-well plates at a cell density of plate so as to achieve 8 X 10⁴ cells/well and cultured overnight at 37°C in a CO₂ incubator at 37°C overnight by use of DMEM medium containing 10% bovine fetal serum. Next On the following day, the culture-medium was removed, and the cells were then washed with serum-free DMEM culture-medium. The above-described In accordance with a protocol attached to lipofectamine (Gibco Co., Ltd.) which was a reagent for transfection, the plasmid DNA which was previously diluted with an OPTI-MEM-culture® medium (Gibco BRL Co., Ltd.) and lipofectamine were mixed with Lipofectamine (a transfection reagent together, manufactured by Gibco BRL Co.) according to the protocol supplied with Lipofectamine. After and after 15-minute minutes incubation, the mixture was added to the cells in each well. The amounts of Lipofectamine DNA and DNA lipofectamine used were, respectively, 1 µg and 4 µl per well, respectively. After 5-hour hours incubation, the culture-medium was removed, and

1 ml of DMEM culture-medium (Gibco BRL Co., Ltd.) containing 10% fetal-bovine fetal serum was added to each well. The plates were incubated for 2-3 days at 37°C and cultured in a CO₂ incubator (5% CO₂) at 37°C for 2 to 3 days. The COS-7 cells transfected obtained after transfection and cultured subsequent culture for 2- to 3 days in this manner were washed with a serum-free DMEM-culture medium. Then, 200 µl of a culture-medium for the binding assay experiment (serum-free DMEM culture-medium containing 0.2% calf-bovine serum albumin, 20 mM Hepes buffer, 0.1 mg/ml heparin, and 0.02% NaN₃) with further containing 20 ng/ml of ¹²⁵I-labeled OCIF was added thereto was added to each well. After culturing for one hour at 37°C Cells were cultured in a CO₂ incubator (5% CO₂), the cells were at 37°C for 1 hour and washed twice with 500 µl of a phosphate buffered saline solution containing 0.1 mg/ml heparin. After washing, 500 µl of 0.1 N NaOH solution was added to each well. The plates were allowed thereto, and then left to stand for 10 minutes at room temperature for 10 minutes so as to lyse the cells. The amount of ¹²⁵I in each well was measured using with a gamma counter (Packard Co., Ltd.). One DNA pool containing cDNA encoding the protein which specifically binds to OCIF was found by After screening a total of the 500 pools. The in total, a DNA pool containing the cDNA encoding a protein that could specifically bind OCIF was further divided isolated. Furthermore, the DNA pools containing the cDNA of the present invention were subfractionated, and then employed to repeat the above-described transfection and screening operations were repeated to isolate the. Thereafter, a cDNA which encodes the encoding a protein which binds could to bind OCIF was isolated. The A plasmid containing this cDNA was named referenced pOBM291. The E. coli containing this plasmid was deposited with the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Biotechnology Laboratory, as pOBM291 on May 23, 1997 under with the deposition No. number of FERM BP-5953. The methods of 5953 on May 23, 1997. Methods for ¹²⁵I-labeling of OCIF with ¹²⁵I and quantitative analysis of determining (the concentration of) ¹²⁵I-labeled OCIF by ELISA are shown as below follows. Labeling of OCIF was ¹²⁵I-labeled in accordance with ¹²⁵I was carried out according to the Iodogen method. Twenty 20 µl of 252.5 mg/ml Iodogen-chloroform solution was added transferred to a 1.5 ml Eppendorf tube, and chloroform was evaporated by heating at 40°C, so as to

prepare an Iodogen-coated tube. ~~The~~After the tube was washed three times with 400 μ l of 0.5 M sodium phosphate buffer (Na-Pi, pH 7.0), and ~~50.5~~ 50.5 μ l of 0.5 M Na-Pi (with a pH of 7.0) was added thereto. Immediately after the addition of 1.3 μ l (18.5 MBq) of Na-¹²⁵I solution (NEZ-033H20, Amersham Co.), Ltd., NEZ-033H20) was added thereto, 10 μ l of 1 mg/ml ~~OCIF~~ OCIF solution (monomer type or dimer type) was added to the tube. After mixing the contents ~~The~~ resulting solution was agitated with a vortex mixer, ~~the tube was allowed~~ and then left to stand at room temperature for 30 seconds. This solution in the tube was transferred to a tube in which 80 μ l of 10 mg/ml potassium iodide, 80 μ l of 0.5 M Na-Pi solution (pH 7.0) and 5 μ l of a phosphate buffered saline solution containing 5% bovine serum albumin (BSA-PBS) ~~werewas~~ previously added and then agitated. After stirring, the mixture ~~This~~ solution was applied to a spin column (1 ml, G-25 fine, manufactured by Pharmacia Co., Ltd.) equilibrated with BSA-PBS, and the column was centrifuging for 5 minutes ~~centrifuged~~ at 2000 ~~2,000~~ rpm for 5 minutes. ~~Four~~After hundred 400 μ l of BSA-PBS was added to the fraction eluted ~~an eluate~~ from the column. After stirring and mixed, 2 μ l of an aliquot of this solution was sampled to measure the ~~subfractionated and its~~ radioactivity by ~~was~~ measured with a gamma counter. The radiochemical purity of the ¹²⁵I-labeled OCIF solution thus prepared ¹²⁵I-labeled OCIF solution was determined by measuring the radioactivity of a fraction precipitated by with 10% TCA. ~~The~~Furthermore, the biological activity as OCIF of the ¹²⁵I-labeled OCIF solution was ~~measured according to the~~ determined in accordance with a method ~~of~~ described in WO 96/26217. ~~The~~Moreover, the concentration of the ¹²⁵I-labeled OCIF was ~~determined~~ measured by the ELISA as follows in the following manner. Specifically ~~That is~~, 100 μ l of 50 mM NaHCO₃ (pH 9.6) in which the 2 μ g/ml of anti-OCIF rabbit polyclonal antibody described in WO 96/26217 was dissolved to a concentration of 2 μ g/ml was added to each well of a 96-well ~~immuno-plate~~ immunoplate (MaxiSorp™, a product of Nunc Co.). ~~The plate was allowed, Ltd., MaxiSorp) and left to stand over night at 4°C overnight.~~ After removing the this solution by ~~was~~ suction removed, 300 ~~200~~ 200 μ l of Block-Ace™ a combined solution of of BLOCKACE (Snow Brand Milk Products Co., Ltd.) and phosphate buffered saline solution (mixing ratio = 25:75); (B-PBSBPB) was added to each well. ~~The plate was and then allowed~~ left to stand for two hours at room temperature for 2 hours. After removing the this solution

by ~~was suction removed~~, the ~~each~~ wells ~~were~~ was washed three times with phosphate buffered saline solution (P-PBS) containing 0.01% Polysorbate 80 (~~P-PBS~~)-80. ~~Next~~ Thereafter, 100 µl of B-PBS containing a ¹²⁵I-labeled OCIF or standard OCIF was added to ~~each well~~. The plate ~~was then allowed thereto and left~~ to stand for two hours at room temperature for 2 hours. After ~~removing the~~ this solution ~~by was suction removed~~, each well was washed six times with 200 µl of P-PBS. ~~One hundred µl of~~ Then, a peroxidase-labeled ~~rabbit anti-OCIF rabbit~~ polyclonal antibody was diluted with B-PBS and 100 µl of the diluted solution was added to each well. ~~The plate was allowed, and then left~~ to stand for two hours at room temperature for 2 hours. After ~~removing the~~ this solution ~~by was suction removed~~, the ~~each~~ wells ~~were~~ was washed six times with 200 µl of P-PBS. Then, 100 µl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well. ~~After incubating the plate and then left to stand~~ at room temperature for 2- to 3 minutes. Thereafter, 100 µl of stopping solution (Stopping Reagent; (Scytek Co., Ltd.) was added ~~to thereto~~. The absorbance of each well. Absorbance at 450 nm of ~~each well~~ was measured using with a microplate reader. The concentration of the ¹²⁵I-labeled OCIF was determined ~~based on~~ from a calibration curve drawn made using the standard ~~preparation of~~ OCIF.

(4) Determination of the nNucleotide sSequence of the cDNA encoding Which Eccodes the entire amino acid sequence Full Length Amino Acid Sequence of OBM

The nucleotide sequence of the OBM cDNA obtained in the Example 8-(3) was determined using a with Taq Dye ~~Deoxy~~ Dye Deoxy Terminator Cycle Sequencing kit (a ~~product of~~ Perkin Elmer Co., Ltd.). Specifically That is, using pOBM291 as a template, the nucleotide sequence of the inserted fragment was directly determined using pOBM291 as a template. ~~Fragments with a length of~~ Furthermore, about 1.0 kb and about 0.7 kb ~~which were~~ fragments obtained by digesting cleaving pOBM291 with a restriction enzyme, EcoRI, were inserted into the EcoRI site of plasmid pUC19 (Takara Shuzo Co., Ltd.) and sequenced, respectively. The nucleotide sequences of these fragments were also determined. The following primers were used: A primer SRR2 which was used to determine nucleotide sequences of for sequencing the DNA fragments inserted into in pcDL-SR ~~ex~~ 296, primers M13 Primer M3 and M13 Primer RV (both manufactured by Takara Shuzo Co., Ltd.) which were used to determine for sequencing the nucleotide

sequences of DNA fragments inserted into the plasmid pUC19, and synthesized a synthetic primer OBM #8 designed based on the nucleotide sequence of OBM cDNA were used. The sequences of these primers are shown as the Sequence SEQ ID No. NOs: 3 to No. 6 in the sequence table 6.

Furthermore, the determined nucleotide sequence of OBM cDNA is shown as Sequence SEQ ID No. NO: 22, and the deduced amino acid sequence determined therefrom is shown as the Sequence SEQ ID No. NO: 1.

<[Example 9]>

Expression of the pProtein encoded by the cDNA of the pPresent invention

Plasmid pOBM291 was transfected into COS-7 cells with lipofectamine in each well of a 6-well plate using Lipofectamine, and the transfected COS-7 cells were cultured for two days in DMEM medium containing 10% fetal bovine serum for 2 days. The medium was replaced with a cysteine-/methionine-free DMEM (Dainippon Seiyaku Co. DAINIPPON PHARMACEUTICAL CO., Ltd. LTD.) (800 µl/well) containing in which 5% dialyzed fetal bovine serum. The (800 µl/well) was added, and the cells were cultured for another 15 minutes. Then, followed by the addition of 14 µl of Express Protein Labeling Mix (NEN CO., LTD., 10 mCi/ml, manufactured by NEN Co.) was added thereto. After culturing the cells were cultured for four hours, 200 µl of DMEM including medium containing 10% fetal bovine serum was added, and the cells were cultured for 1 hour. After one hour culturing, the cells were washed twice with PBS. Then, 0.5 ml of a-TSA buffer (10 mM Tris-HCl (pH 8.0) containing 0.14 M NaCl, and 0.025% NaN₃), containing 1% Triton X-100, 1% bovine hemoglobin, 10 µg/ml leupeptin, 0.2 TIU/ml aprotinin, and 1 mM PMSF, was added, and the mixture was allowed cells were left to stand for one hour on ice for 1 hour. Cells After the cells were disrupted crushed by pipetting, centrifugation was carried out at 3,000 xg at 4°C and centrifuged at 3000 x g for 10 minutes at so 4°C as to obtain a supernatant. To 100 µl of this supernatant, 200 µl of dilution buffer (TSA buffer containing 0.1% Triton X-100, 0.1% bovine hemoglobin, 10 µg/ml leupeptin, 0.2 TIU/ml aprotinin, and 1 mM PMSF) was added to 100 µl of this, and the resulting supernatant. The mixture was shaken for one hour at 4°C together with pProtein A Sepharose® (50 µl). The resultant

mixture was at 4°C for 1 hour, and then centrifuged at 1,500 X g for one minute at 4°C to collect a supernatant. Thereby, and thereby a fraction(s) which is non-specifically adsorbed nonspecifically binding to the Protein A Sepharose® was removed. OCIF (1 µg) was added to this supernatant, and the mixture obtained supernatant was shaken at 4°C for one hour to achieve the binding of so that OBM bound OCIF to OBM. Anti Then, an anti-OCIF polyclonal antibody (50 µg) was added, and the mixture solution was shaken at 4°C for one hour at 4°C. Then, Protein A Sepharose® (10 µl) was further added, and the mixture solution was further shaken at 4°C for another additional 1 hour at 4°C, followed by centrifuge. The solution was centrifuged at 1,500 xg at 4°C for 1 minute at 4°C to collect and the precipitated fraction was collected. The precipitate. The precipitate resulting from centrifugation at 1,500 xg at 4°C for 1 was washed twice with the dilution buffer, twice with a the dilution buffer without bovine hemoglobin-free dilution buffer, once with TSA buffer, and once with 50 mM Tris-HCl (pH 6.5). After washing, SDS buffer (0.125 M Tris-HCl, 4% dodecyl sodium dodecylsulfate, 20% glycerol, 0.002% Bromophenol Blue, pH 6.8) containing 10% β-β mercaptoethanol was added to the precipitate. The mixture precipitate was heated at 100°C for 5 minutes at 100°C and subjected to SDS-PAGE (12.5% polyacrylamide gel, Daiichi Chemical Pure Chemicals Co., Ltd.). The After the gel was fixed according to in accordance with a conventional commonly used method. Isotope, signals of isotope were amplified using with Amplify™ (Amersham Co., Ltd.), and the sample fixed gel was exposed to Bio-Max BioMax® MR film (KODAK Kodak Co., Ltd.) at -80°C. The results are shown in Figure 8, which indicates that Fig. 8. The molecular weight of the protein encoded by the cDNA of the present invention has a molecular weight of was found to be about 40,000.

<[Example 10]>

Binding of the pProtein_eEncoded by the cDNA of the pPresent iInvention to OCIF

Plasmid pOBM291 was COS-7 cells were transfected into COS cells with plasmid pOBM291 with lipofectamine in each well wells of a 24-well plate using Lipofectamine. After culturing and cultured for 2- to 3 days. Then, the cells were washed with serum-free DMEM culture medium.—, and 200 µl of culture medium for the binding assay experiment (serum-free DMEM culture medium containing 0.2% calf bovine serum;

albumin, 20 mM Hepes buffer, 0.1 mg/ml heparin, and 0.2% NaN₃), supplemented with containing 20 ng/ml of ¹²⁵I-labeled OCIF, was added to the wellsthereto. To the other wellsFurthermore, 200 µl of culturethe medium for the binding assay to whichexperiment containing 8 µgµg/ml of unlabelled OCIF-had been added, in addition to 20 ng/ml of the ¹²⁵I-labeled OCIF, -was added was added to other wells. After culturing for one hour at 37°CThe cells were cultured in a CO₂ incubator (5% CO₂), the cells were at 37°C for 1 hour, and washed twice with 500 µl of phosphate buffered saline solution containing 0.1 mg/ml of heparin. Then. After washing, 500 µl of 0.1 N NaOH solution was added to each well, and then the platewell was allowedleft to stand for 10 minutes-at room temperature for 10 minutes so as to dissolve lyse the cells. The amount of ¹²⁵I in each well was measured bywith a gamma counter. As a result, as shown in Figure 9, it was confirmed that the ¹²⁵I-labeled OCIF was found to bindbound only to the cells in whichtransfected with the plasmid pOBM291-was transfected291 as shown in Fig. 9. In additionFurther, it was also confirmed that the binding was confirmed to be conspicuouslysignificantly inhibited by the addition of (unlabeled) OCIF at a 400-fold higher concentration of (unlabeled) OCIF. TheseFrom these results have demonstrated, it was revealed that the OBM, a protein-OBM encoded by the cDNA onof the plasmid pOBM291291 specifically binds tobound OCIF on the surface of the transfected COS-7 cells.

<[Example 11]>

Crosslinking Experiment of ¹²⁵I-labeled OCIF and to the pProtein. eEncoded by the cDNA of the pPresent. iInvention

Crosslinking of ¹²⁵I-labeled monomer type OCIF and the protein encoded by the eDNA of the present invention was carried outIn order to investigateanalyze the characteristics of the protein encoded by the cDNA of the present invention in further detailmore specifically, ¹²⁵I-labeled monomer type OCIF was allowed to crosslink with the protein encoded by the cDNA of the present invention. After transfection of COS-7 cells were transfected with plasmid pOBM291 into COS-7 cells according toin accordance with the method used- described in the Example 8 -(3), 200 µl of the culture medium for the binding assay, as described above, experiment containing the above ¹²⁵I-labeled OCIF (25 ng/ml) was added to the wellsthereto. TheFurthermore, culturethe

medium for the binding assay to which unlabeled OCIF at experiment containing a 400-fold higher concentration was added of unlabeled OCIF in addition to the 125 I-labeled OCIF was added to the other wells. After culturing for one hour at 37°C The cells were cultured in a CO₂ incubator (5% CO₂), the cells were at 37°C for 1 hour and washed twice with 500 µl of phosphate buffered saline solution containing 0.1 mg/ml of heparin. Five hundred 500 µl of phosphate buffered saline solution containing 100 µg/ml of a crosslinking agent, DSS (dDisuccinimidyl suberate, manufactured by Pierce Co., Ltd.) was added to these cells, followed by a reaction and the cells were allowed to react at 0°C for 10 minutes at 0°C. The After the reaction, the cells in these wells were washed twice with 1 ml of cold phosphate buffered saline (solution cooled to 0°C). After the addition of Then, 100 µl of 20 mM Hepes buffer containing 1% Triton X-100 (Wako Pure Chemicals Co. Chemical Industries, Ltd.), 2 mM PMSF (Pphenylmethylsulfonyl fluoride, Sigma Co.), 10 µM Pepstatin (Wako Pure Chemicals Co., Ltd.), 10 µM Leupeptin pepstatin (Wako Pure Chemicals Co. Chemical Industries, Ltd.), 10 µM leupeptin (Wako Pure Chemical Industries, Ltd.), 10 µM antipain (Wako Pure Chemicals Co. Chemical Industries, Ltd.) and 2 mM EDTA (Wako Pure Chemicals Co. Industries, Ltd.) was added to these cells, and the wells were allowed left to stand for 30 minutes at room temperature for 30 minute so as to dissolve the cells. Fifteen After 15 µl aliquots of these samples were heated in the presence of treated with SDS under non-reducing conditions according to in accordance with a conventional commonly used method and, they were subjected to electrophoresis with gel for SDS-electrophoresis using (gradient of 4- to 20% polyacrylamide gradient gel (Daiichi Pure Chemical Co., DAIICHI PURE CHEMICALS CO., Ltd LTD.). After the electrophoresis, the gel was dried and exposed for 24 hours at -80°C to a BioMax® MS film (Kodak Co., Ltd.) using a with BioMax® MS sensitization amplifying screen (Kodak Co., Ltd.) at -80°C for 24 hours. The exposed films was were developed according to in accordance with a conventional commonly used method. As a result, a band with a molecular weight of a range of 90,000-110,000, shown in Figure 10, was detected by results of crosslinking of the 125 I-labeled monomer type OCIF and with the protein encoded by the cDNA of the present invention, a band having a molecular weight of about 90,000 to 110,000 was detected as shown in Fig. 10.

<[Example 12]>

Northern blotting-Blot aAnalysis

ST2 cells were cultured to become confluent in a 25 -cm² T flask, for ~~attached~~ cell cultures ~~were~~ culturing adherent cells, and treated with trypsin and. After being stripped from the T flask, ~~After washing~~, the cells were washed and seeded into a 225 -cm² T flask ~~and cultured for 4 days in a CO₂ incubator with~~ 60 ml of an α -MEM culture medium containing 10⁻⁸ M of the active-form of vitamin D₃ 10⁻⁷ M dexamethasone, and 10% bovine fetal bovine serum was added thereto, and the cells were cultured in a CO₂ incubator for 4 days. ~~Total~~ Then, total RNA was extracted from the above-cultured ST2 cells using with ISOGEN (Wako Pure Chemicals Co. Industries, Ltd.). ~~The~~ In addition, total RNA was also extracted ~~in the same manner from ST2 cells which were cultured in the absence of the active-form of vitamin D₃ and dexamethasone.~~ After in accordance with the addition of above method. To 20 μ g (4.5 μ l) of each total RNA sample, 2.0 μ l of 5X gel electrophoresis buffer solution (0.2 M morpholine propane sulfoniemorpholinopropanesulfonic acid, (pH 7.0, 7.0), 50 mM sodium acetate, 5 mM EDTA) and, 3.5 μ l of formaldehyde, and 10.0 μ l of formamide to 20 μ g (4.5 μ l) of each of the total RNAs, the mixtures were added. The total RNA samples were incubated at 55°C for 15 minutes at 55°C and subjected to electrophoresis. The gel Gels for electrophoresis was prepared according to the formulation consisted of 1.0% agarose, 2.2 M deionized ionized formaldehyde, 40 mM morpholinopropane sulfoniemorpholinopropanesulfonic acid (pH 7.0), 10 mM sodium acetate, and 1 mM EDTA. The Moreover, the electrophoresis was carried out performed in a buffer solution of comprising 40 mM morpholine propane sulfoniemorpholinopropanesulfonic acid, (pH 7.0, 7.0), 10 mM sodium acetate, and 1 mM EDTA. After the electrophoresis, the RNA was transferred ~~ontoto~~ onto nylon membranes. About 1.0 kb DNFA fragments ~~was were~~ obtained by digesting cleaving pOBM291 with a restriction enzyme, EcoRI. Hybridization was carried out using this DNA fragment, and labeled with a Megaprime DNA labeling kit (Amersham Co.) and α -³²P-dCTP (Amersham Co.), Ltd.) using MEGAPRIME DNA Labeling Kit (Amersham Co., Ltd.), and thus used as a probe probes for hybridization. As a result, as shown in Figure 11, it was confirmed revealed that when ST2 cells were cultured in the presence of active form vitamin D₃ and dexamethasone,

gene expression of the protein (OBM) encoded by the cDNA of the present invention (OBM) was isstrongly induced strongly in the ST2 cells cultured in the presence of the active-form of vitamin D₃ and dexamethasone.

<[Example 13]>

Osteoclasts formation supporting capabilityAbility of the pProtein eEncoded by the cDNA of the present inventionPresent Invention to Support Osteoclast Formation

pOBM291 was transfected into COS cells according to-In accordance with the same method described in the-Example 8(3)-(3), COS-7 cells were transfected with pOBM219. After three3-day daysincubation, trypsinizedthe cells were treated with trypsin and then centrifuged-washed once with phosphate buffered saline solution-by centrifugation. Then, thenthe cells were fixed withat room temperature for 5 minutes in suspension of PBS containing 1% paraformaldehyde at room temperature for 5 minutes, followed by washing with PBSand then centrifuged-washed six times bywith centrifugationPBS. 700 μ l of 1×10^6 /ml mouseMouse spleen cells and 350 μ l of 4×10^4 /ml-ST2 cells which-were suspended in-aprepared with α -MEM culture-medium containing 10^{-8} M of the active-form of vitamin D₃, 10^{-7} M dexamethasone, and 10% bovine fetal bovine-serum,-were so that the cell concentration become 1×10^6 cells/ml or 4×10^4 cells/ml and then added to a 24-well plate- in a volume of 700 μ l and 350 μ l, respectively. Furthermore, TC insert (Nunc Co., Ltd.) was set in each well. The above-described-fixed COS cells (350 μ l) diluted to various concentrationsstepwise with the above-mentioned culture medium and OCIF solution-(50 μ l), were added to the TC inserts and cultured at 37°C for 6 days-at 37°C. As a result, it was confirmedrevealed that the osteoclasts formation inhibitivean activity of OCIF can-be-inhibitedto inhibit osteoclast formation was suppressed by the protein encoded by the cDNA of the present invention.

<[Example 14]>

Expression of sSecretedory-form-type OBM

(1) Construction of a-plasmidPlasmid for the-expression-of-secretedExpressing Secretory-formType OBM Expression

A PCR reaction was carried out using OBM HF (Sequence-Table, SequenceSEQ ID No.-NO: 7)-and-/OBM XR (Sequence-Table, SequenceSEQ ID No.-NO: 8) and

pOBM291 as primers and pOBM291 as a template, respectively. After purification by the reaction product was purified through agarose gel electrophoresis, the product was it was digested with the restriction enzymes, HindIII and EcoRI, and further then purified by through agarose gel electrophoresis again. The purified fragment (0.6 kb), Hind III/HindIII/EcoRI fragment (5.2 kb) of pSec TagA (Invitrogen Co., Ltd.) and EcoRI/PmaeCI fragment (0.32 kb) of OBM cDNA were ligated using a was subjected to ligation kit verusing Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.), and subsequently *E. Escherichia coli* DH5- α was transformed using by the reaction ligation product. Plasmids werewas purified by means of alkali-SDS method from the resulting obtained ampicillin - resistant strains and digested by alkaline-SDS method and then cleaved with restriction enzymes so as to select a plasmid with fragments of a length of wherein 0.6 Kbbk and 0.32 kb being of fragments were inserted into pSec TagA. Selected The selected plasmid was identified as having a subjected to sequencing with Dye Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.), thereby it was confirmed that the plasmid had the sequence encoding the secreted secretory-form type OBM (nucleotide sequence: nucleotides 338-1355 in of Sequence SEQ ID No: NO: 2, amino acid sequence: acids 72-316 in the Sequence of SEQ ID No: NO: 1) by sequencing using a dyeterminator cycle sequencing FS kit (Perkin Elmer Co.). This After the plasmid was digested with restriction enzymes, NheI and XhoI to isolate, a fragment (1.0 kb) containing the corresponding to secreted ory-form type OBM cDNA was collected by agarose gel electrophoresis. This fragment was inserted into thea NheI/XhoI fragment (10.4 kb) of and digested expression vector, pCEP4 (10.4 kb) (Invitrogen Co., Ltd.), using a, by using the ligation kit, and *Escherichia E. coli* DH5- α was α were transformed using with the reaction ligation product thereof. Plasmids werewas purified by alkali-SDS method, from the resulting ampicillin -resistant strains obtained, by alkaline-SDS method, and digested with the restriction enzymes. Then the plasmid was analyzed so as to select an *Escherichia E. coli* strain having the secreted form strains which had a plasmid for expressing secretory-type OBM expression plasmid (pCEP sOBM) with the eorreet desired structure. The An *Escherichia E. coli* strain containing having the pCEP sOBM was cultured, and the pCEP sOBM was purified using therefrom with QIA-filter plasmid midi kit® Filter Plasmid Midi Kit (QIAGEN CoCO., LTD.).

(2) Expression of sSecretedory-formType OBM

293-EBNA cells were suspended in IMDM containing 10% FCS (IMDM-10% FCS), and seeded ~~into in a collagen-coated 24- well plate-coated with collagen~~ (manufactured by Sumitomo Bakelite Co SUMITOMO BAKELITE CO., LtdLTD.) ~~in aso~~ that the cell density ~~concentration of~~ was 2×10^5 cells/2 ml/well, and cultured overnight. The cells were transfected with 1 μ g of pCEP sOBM or pCEP4 using 4 μ l of ~~Lipofectamine (Gibco Co.)~~. ~~After culturing for two days (GIBCO CO., LTD.), and then~~ cultured in 0.5 ml of a-serum-free IMDM or IMDM-10% ~~FCS FCS~~ for another 2 days. ~~Thereafter,~~ the conditioned medium was collected. Expression of ~~the secretedsecretory-~~ formtype OBM in the conditioned medium was confirmed as follows ~~in the following~~ manner. ~~Sodium hydrogen carbonate~~ After sodium hydrogencarbonate was added to the conditioned medium ~~to aso that the final concentration of~~ was 0.1 M ~~and,~~ the culture solution was added to a 96-well plate. ~~The plate was allowed,~~ and left to stand at 4°C overnight ~~at 4°C, thereby immobilizing.~~ Then the OBM in the conditioned medium ~~on~~ was immobilized in the 96-well plate. The plate ~~was filled with a Block Ace™~~ This plate was left to stand for blocking at room temperature for 2 hours by use of BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution diluted ~~four fold~~ with PBS ~~to be one forth concentration~~ (B-PBS) ~~and allowed to stand for two hours at room~~ temperature to block residual binding sites of the plate. ~~After the addition to each well of~~ Then, 100 μ l of 3-100 ng/ml of OCIF which was diluted with B-PBS, the plate was ~~allowed~~ was added to each well, and the wells were left to stand for two hours at 37°C, ~~followed by for 2 hours.~~ After washing the plate with PBS containing 0.05% Tween 20 (PBS-T). ~~Then, 100, 100 μ l of a peroxidase-labeled rabbit-anti-OCIF rabbit polyclonal antibody, which was described in WO 96/26217 which was 26217,~~ diluted with B-PBS was added to each well. ~~After allowing,~~ and the cells were left to stand at 37°C for ~~two 2~~ hours ~~at 37°C, the wells were washed six times.~~ After washing each well with PBS-T. ~~Then six times,~~ a 100 μ l of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added ~~in the amount of 100 μ l per well~~ thereto and allowed ~~then left~~ to stand at room temperature for about 10 minutes. ~~Thereafter, whereupon the reaction~~ was terminated by the addition of 100 μ l of a termination solution (Stopping Reagent, (Scytek Co., Ltd.) was added to each well. Absorbance The absorbance of each well at

450 nm of each well was measured by with a microplate reader. The results are shown in Figure 12. In the plate in which indicates that substances included in the absorbance conditioned medium of the cells transduced by pCEP sOBM were immobilized, absorption at 450 nm increased according to the OCIF concentration of the added OCIF in dependent manner. On the other hand, in the plate in which substances included in the conditioned medium of of the cells transfected with pCEP sOBM was immobilized. On the other hand, transduced by pCEP4 vector were immobilized, no increase in absorbance absorption at 450 nm was seen in the plate in which the conditioned medium of the cells transfected with vector pCEP4 was immobilized observed. Figure Furthermore, Fig. 13 shows the results of an experiment wherein experiments when the proportion amount of the conditioned medium which is used for applied to the immobilization was changed varied within a range of 5- to 90% and a specific constant concentration of OCIF (50 ng/ml) was further added. It can be seen that in the absorbance plate in which substances included in the conditioned medium of the cells transduced by pCEP sOBM were immobilized, absorption at 450 nm increased according corresponding to the an increase in the amount of the proportion of conditioned medium. On the conditioned medium in other hand, in the plate wherein plate in which substances included in the conditioned medium of of the cells transfected with pCEP sOBM was immobilized, whereas transduced by pCEP4 vector were immobilized, no such increase in absorbance was seen in the plate wherein the conditioned medium of the cells transfected with vector pCEP4 was immobilized absorption was observed. From these results, it secretory-type OBM was confirmed that secreted form OBM is to be produced in the conditioned medium of the cells transfected with by pCEP sOBM.

<[Example 15]>

Expression of Thioredoxin-OBM Fusion pProtein (Trx-OBM)

(1) Construction of a thioredoxin Vector for Expressing Thioredoxin-OBM Fusion pProtein (Trx-OBM) expression vector

Ten 10 µl of 10X ExTaq buffer (Takara Shuzo Co. TAKARA SHUZO CO., LTD.), 8 µl of 10 mM dNTP (Takara Shuzo Co. dNTPS (TAKARA SHUZO CO., LTD.), 77.5 µl of sterilized distilled water, 2 µl of an aqueous pOBM291 solution of

pOBM291 (10 ng/ μ l), 1 μ l of primer OBM3 (100 pmol/ μ l, Sequence Table, Sequence SEQ ID No. NO: 9), 1 μ l of primer OBMSalR2 (100 pmol/ μ l, Sequence Table, Sequence SEQ ID No. NO: 10), and 0.5 μ l of ExTaq (5-u/ μ l) (Takara Shuzo Co., Ltd.) were mixed together, and reacted then (PCR reaction) was conducted in a microtube-centrifugal tube for centrifugation. After reacting the reaction was carried out at 95°C for 5 minutes, at 50°C for one second, at 55°C for one minute, at 74°C for one second, and at 72°C for 5 minutes, at the cycle reaction consisting of a reaction at 96°C for one minute, at 50°C for one second, at 55°C for one minute, at 74°C for one second, and at 72°C for 3 minutes, was repeated 25 times. From the total reaction liquid After gel electrophoresis through 1% agarose, an approximately 750 bp DNA fragment of about 750 bp was purified by 1% agarose gel electrophoresis using from the whole reaction solution with QIAEX® II gGel eExtraction kKit (QIAGEN Co., Ltd.). The whole amount All of the purified DNA fragment was digested cleaved with restriction enzymes SalI and EcoRI (Takara Shuzo Co., Ltd.), and subjected to an 1.5% agarose gel electrophoresis to purify a DNA fragment of about 160 bp (Fragment 1), which was (fragment 1) was purified by gel electrophoresis through 1.5% agarose and dissolved in 20 μ l of sterilized distilled water. In the same manner Similarly, a DNA fragment of about 580 bp (Fragment 2) obtained by digesting 4 μ g of pOBM291 and 2 μ g of pTrxFus (Invitrogen Co., Ltd.) were cleaved with restriction enzymes BamHI and BamHI/EcoRI and BamHI/SalI (Takara Shuzo Co., Ltd.) and a, respectively. A DNA fragment of about 580-bp (fragment 2) and an approximately 3.6 -kb DNA fragment (Ffragment 3) obtained by digesting 2 μ g of pTrXFus (Invitrogen Co.) with restriction enzymes BamHI and SalI (Takara Shuzo Co.) were respectively purified therefrom, respectively, and dissolved in 20 μ l of sterilized distilled water. The QIAEXII-gel extraction kit QIAEX® II Gel Extraction Kit was used for for purifying the purification of DNA fragments. Fragments 1-1, 2 and 3 were ligated by incubating them using DNA Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.) at 16°C for 2.5 hours using DNA ligation kit ver. 2 (Takara Shuzo Co.). Using the ligation reaction liquid Then, *Escherichia coli* strain-GI724 cells (Invitrogen Co., Ltd.) was were transformed according to with the ligation product in accordance with the method described in the Instruction Manual of an instruction manual attached to ThioFusion Expression System (Invitrogen Co., Ltd.). A

~~microorganism strain with~~ Among the resulting ampicillin-resistant transformants, one ~~having a plasmid, in which the an~~ OBM cDNA fragment (nucleotide sequence: -350-
-1111 ~~in the Sequence of SEQ ID No. NO: 2, amino acid sequence corresponding to: 76-~~
316 ~~in the Sequence of SEQ ID No. NO: 1) is fused in frame~~ was linked to a thioredoxin
gene ~~in the same reading frame and~~ was selected ~~from the resulting ampicillin-resistant~~
transformants ~~by the after~~ analysis of ~~restriction maps~~ DNA fragment map obtained by
digestion ~~with restriction enzymes~~ enzyme cleavage and DNA sequence
determination ~~sequencing~~. The ~~microorganism obtained~~ strain thus obtained was
named ~~referenced~~ as GI724/pTrxOBM25.

(2) Expression of OBM in *Escherichia coli*

The GI724/pTrxOBM25 strain and the GI724 containing strain having pTrxFus
(GI724/pTrxFus) were respectively cultured ~~six hours with shaking at 30°C~~ in 2 ml of
RMG-Amp culture medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl,
1.2% casamino acid (Difco Co., Ltd.), 1% glycerol, 1 mM MgCl₂, and 100 µg/ml
ampicillin (Sigma Co., Ltd.), pH 7.4) ~~with shaking at 30°C for 6 hours~~. ~~The broth~~ 0.5 ml
of the ~~broth~~ cell suspension was added to 50 ml of Induction culture medium (0.6%
Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 0.2% casamino acid, 0.5% glucose,
1 mM MgCl₂, 100 µg/ml ampicillin, pH 7.4) and cultured with shaking at 30°C. ~~When~~
OD_{600nm} ~~reached about 0.5, L-tryptophan was added to so that the final concentration~~
~~of was~~ 0.1 mg/ml, ~~followed by shaking when the culture value at OD₆₀₀ became about 0.5,~~
~~and the cells were further cultured at 30°C for an additional 6 hours~~. The culture cell
~~broth~~ suspension was centrifuged at 3000 ~~3,000~~ × g to collect ~~and the collected cells,~~
which were ~~then~~ suspended in 12.5 ml of PBS (10 mM ~~phosphate~~ phosphoric acid buffer,
0.15 M NaCl, pH 7.4). The suspension was subjected to ~~an ultrasonic~~
generator ~~ultrasonication using a ultrasonicator~~ (Ultrasonics Co., Ltd.) ~~to so disrupt that the~~
cells. ~~The disrupted cells were crushed and then centrifuged at 7000~~ × 7,000 X g for 30
minutes ~~to obtain a~~. The recovered supernatant liquid ~~was used~~ as a soluble protein
fraction. ~~Ten~~ 10 µl of this soluble protein fraction solution was subjected to SDS
polyacrylamide (10%) electrophoresis under reducing conditions. As a result, a band
~~with having~~ a molecular weight of about 40 kDa ~~which was observed in the soluble~~
protein fraction solution of GI724/pTrxOBM25, while not detected ~~observed~~ in the

soluble protein fraction solution of GI724/pTrxFus was found in the soluble protein fraction of GI724/pTrxOBM25 (Figure Fig. 14). Accordingly, it was confirmed that the thioredoxin-OBM fusion protein (Trx-OBM) of thioredoxin and OBM was expressed in *Escherichia coli*.

(3) Binding capability Ability of Trx-OBM to OCIF

Binding of In the following experiment, it was confirmed that the expressed Trx-OBM bound to OCIF ~~was confirmed according to the following experiment.~~ Anti-thioredoxin antibody (Invitrogen Co., Ltd.) ~~which was diluted to 5000-fold with 10 mM sodium hydrogen carbonate~~ hydrogencarbonate solution so that the concentration was 1/5,000. 100 µl thereof was added to each well of a 96-well immunoplate (Nunc Co., Ltd.) in the amount of 100 µl per well. ~~After being allowed and then left to stand at 4°C overnight at 4°C, the liquid in the wells. After the solution in each cell was discarded.~~ Two hundred, 200 µl of a solution prepared by diluting Block Ace™ of 1/2 concentration of BLOCKACE (Snow Brand Milk Products Co., Ltd.) two-fold diluted with PBS (BA-PBS) was added to each well. ~~After being allowed and then left to stand for one hour at room temperature, for 1 hour. After the solution was discarded and, 100 µl of the soluble protein fractions originating fraction solution derived from the above-described GI724/pTrxOBM25 or which was diluted stepwise with BA-PBS and 100 µl of that derived from GI724/pTrxFus, each which was diluted stepwise with BA-PBS in various concentrations~~ BPB were added to each well in the amount of 100 µl. After being allowed wells and left to stand for two hours at room temperature, for 2 hours, respectively. After washing each well was washed well three times with PBS-T and charged with, 100 µl of OCIF (100 ng/ml) which was diluted with BA-PBS. After being allowed was added to each well and left to stand for two hours at room temperature, each for 2 hours. After washing each well was washed three times with PBS-T and charged with, 100 µl of peroxidase- labeled rabbit-anti- OCIF rabbit polyclonal antibody (described described in WO 96/26217) 26217, which was diluted 2,000-fold with BA-PBS. After being allowed so that the concentration was 1/2,000, was added to each well and left to stand for two hours at room temperature, each for 2 hours. After washing each well was washed six times with PBS-T and charged with, 100 µl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co.). After being

allowed, Ltd.) was added thereto and then left to stand at room temperature for about 10 minutes at room temperature. Thereafter, each well was charged with 100 µl of termination solution (Stopping Reagent, (Scytec Co., Ltd.) was added thereto. Absorbance of each well at 450 nm was measured by with a microplate reader. The results are shown in Figure Fig. 15. When the concentration of the soluble protein fraction solution derived from GI724/pTrxFus increased, the absorbance increased in a concentration (of the added solution)-dependent manner, while no difference in absorbance was observed between the sample with when the soluble protein fraction originating solution derived from GI724/pTrxFus was added thereto and the sample without the addition of this when said soluble protein fraction solution was not added. On the other hand Furthermore, the absorbance increased in the samples to which the soluble protein fraction originating from GI724/pTrxOBM25 was added in proportion to the concentration of the soluble protein fraction Fig. The 16 shows the results of the other experiment wherein experiments when the dilution rate of the soluble protein fraction solution was maintained kept constant (1%) while adding and OCIF diluted stepwise with BA-PBS in different concentrations (0-100 ng/ml) are shown in Figure 16. was further added. It can be seen that the absorbance remained Absorbance was kept low at any concentrations regardless of the concentration of OCIF in samples using a when soluble protein fraction originating solution derived from GI724/pTrxFus was added. However, whereas the absorbance was increased in proportion to the an OCIF concentration in the samples to which the dependent manner when soluble protein fraction originating solution derived from GI724/pTrxOBM25 was added. Based on these results Thus, it was confirmed that the Trx-OBM which is produced from in GI724/pTrxOBM25 has a capability of binding had an ability to bind OCIF.

(4) Large Scale cultivation Culture of Escherichia E. coli which produces Producing Trx-OBM

GI724/pTrxOBM25 cells were was spread on an RMG-Amp agar medium (0.6% Na₂PO₄HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 2% casamino acid, 1% glycerol, 1 mM MgCl₂, 100 µg/ml ampicillin, 1.5% agar, pH 7.4) using with a platinum transfer loop. The cells were and cultured at 30°C overnight at 30°C. The cultured cells were suspended in 10 ml of Induction medium. The 5 ml of the suspension was added 5 ml for

each to two of 2-l Erlenmeyer L conical flasks containing 500 ml of Induction medium and cultured at 30°C with by shaking at 30°C. When the OD_{600nm} reached about 0.5, L-tryptophan was added to so that the final concentration of was 0.1 mg/ml. Culturing with when OD₆₀₀ value became about 0.5, and then the cells were further cultured by shaking was at continued 30°C for six 6 hours at 30°C. The culture cell broth suspension was centrifuged at 3,000 X g for 20 minutes at 3000 x g to collect, and the cells, which were collected and then suspended in 160 ml of PBS. The suspension was subjected to ustrasoniaction using an ultrasonic generator ultrasonicator (Ultrasonics Co., Ltd.) to disrupt the for crushing cells. The supernatant liquid was, and then centrifuged at 7,000 X g for 30 minutes at 7000 x g to obtain a. Thereafter, the supernatant was recovered as soluble protein fraction.

(5) Preparation of OCIF-immobilized aAffinity eColumn

Two 2 g of TSKgel AF-Telresyl Toyopal TOYOPAL 650 (Tosoh Corp Toso Co., Ltd.) and 40 ml of 1.0 M potassium phosphate buffer (pH 7.5) containing 35.0 mg of recombinant OCIF, which was prepared according to the by a method described in WO 96/26217, 26217 were mixed. The mixture was together and gently shaken at 4°C overnight at 4°C to effect so as to cause a coupling reaction. The reaction mixture was centrifuged to remove After the supernatant. To inactivate excess active residues was removed by centrifugation, 40 ml of 0.1 M Tris-HCl hydrochloric acid buffer (pH 7.5) was added to the precipitated carrier, and the mixture was gently shaken at room temperature for one 1 hour. The carrier, in a column was washed order to inactivate an excess amount of active groups thereon. After washing the column with both 0.1 M glycine-HCl hydrochloric acid buffer (pH 3.3) containing 0.01% Polysorbate 80 and /0.2 M NaCl and 0.1 M sodium citrate buffer (pH 2.0) containing 0.01% Polysorbate 80 and /0.2 M NaCl. The carrier in the column was equilibrated by charging, the column was washed twice with 10 mM sodium phosphate buffer (pH 7.4) containing 0.01% Polysorbate 80 80 and equilibrated therewith.

(6) Purification of Trx-OBM aUsing OCIF-iImmobilized aAffinity eColumn

Unless otherwise indicated, purification Purification of Trx-OBM was carried out at 4°C unless otherwise stated. The above-mentioned OCIF-immobilized affinity carrier (10 ml) and the above soluble protein fraction solution (120 ml) prepared described in

Example 15-(4) were mixed together. The mixture was gently shaken overnight at 4°C in four 50- ml centrifuge tubes using with a rotor at 4°C overnight. ~~An~~The carrier in the mixture was embedded in Econo-column™ Column (Bio-Rad Co., Ltd., internal diameter: 1.5 cm, length: 15 cm, manufactured by BioRad Co.) was filled with the carrier in the mixture. The column was charged washed with 300 ml of PBS containing 0.01% Polysorbate 80, 100 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 0.01% Polysorbate 80 and 2 M NaCl, and 100 ml of 0.1 M glycine-HCl hydrochloric acid buffer (pH 3.3) containing 0.01% Polysorbate 80 and 0.2 M NaCl, in that order. Next, proteins adsorbed in ~~Then~~, protein was eluted from the column were eluted with 0.1 M sodium citrate buffer (pH 2.0) containing 0.01% Polysorbate 80 and 0.2 M NaCl. The 5 ml eluate was fractions were collected in 5-ml portions. Each fraction thus collected was immediately neutralized with addition Immediately, a 10% volume of 2 M Tris buffersolution (pH 8.0) was added for neutralization. PresenceThe presence or absence of Trx-OBM in each fraction of the eluted fractions eluate was determined according to examined in accordance with the above method previously as described in Example 15-(3) (the binding capability ability to bind OCIF). The fractions Fractions containing Trx-OBM were collected and further purified further.

(7) Purification of Trx-OBM by gGel fFiltration

AboutUsing Centriplus® 10 and Centricon® 10 (Amicon Co., Ltd.), about 25 ml of the above Trx-OBM fractions obtained in fraction of Example 15 -(6) was concentrated by centrifugation to a final volume of about 0.5 ml by centrifuge using Centriplus 10 and Centricon 10 (Amicon Co.). This sample was applied subjected to a Superose® 12 HR 10/30 column (1.0 x 30 cm, Pharmacia Co., Ltd.) previously equilibrated with PBS containing 0.01% Polysorbate 80. For the separation, The column was developed using PBS containing 0.01% Polysorbate 80 was used as a mobile phase at a flow rate of 0.25 ml/min. The and 0.25 ml eluate fractions were collected from the column was collected in 0.25-ml portions. The Trx-OBM in the thus collected fractions was detected by the same method as previously described in Example 15-(3) and by SDS-polyacrylamide electrophoresis (gradient gel of 10- to 15% polyacrylamide-gel, Pharmacia Co., Ltd.) using Phast System (Pharmacia Co., Ltd.) and silver staining. Fractions (Fr. 20- to 23) containing purified Trx-OBM were collected and the subjected to measurement of Trx-

OBM protein concentration of Trx-OBM was determined. The measurement of the protein concentration was carried out with DC-protein assay kit (Bio-Rad Co., Ltd.) using bovine serum albumin as a standard substance using DC Protein assay kit (BioRad Co.).
<[Example 16]>

Osteoclast formation inducing activity of OBM

Osteoclastogenesis Promoting Activity of OBM

COS-7 cells were transfected with pOBM291 and pcDL-SR α 296 were respectively transfected into COS-7 cells using Lipofectamine (Gibco BRL Co., Ltd.), respectively. The After the cells were cultured in DMEM containing 10% FCS for one day, trypsinized they were treated with trypsin, plated on and seeded in a 24-well plate, in which a cover slip glass (15 mm round shape, manufactured by Matsunami Co., Ltd.) in 24-well plates at was seated, so that the concentration became 5×10^4 cells-per/-well, and. The cells were then cultured for 2 another two days. The After washing the culture plate was washed once with PBS. The cells were fixed with, PBS containing 1% paraformaldehyde was added thereto, and the cells were incubated at room temperature for 8 minutes and fixed. The After washing the plate on in which the fixed cells were attached was washed 6 fixed six times with PBS, then 700 μ l of 1×10^6 cells/ml suspension of mouse spleen cells suspended at 1×10^6 /ml cell in α -MEM containing 10^{-8} M of the active-form of vitamin D₃, 10^{-7} M dexamethasone, and 10% bovine fetal bovine serum werewas added to each well cell. A Millicell® PCF (Millipore Co., Ltd.) was set in on each well, and a 700 μ l of 4×10^4 cells/ml suspension of ST2 cells in the above-mentioned culture medium (4×10^4 /ml) werewas added, 700 μ l per well, into to the Millicell® PCF followed by incubation and cultured at 37°C for 6 days. After the culture, the Millicell® PCF was removed, and the plate was washed once with PBS, and the. The cells were fixed with acetone-ethanol solution (50:50) for one minute. Then, and then only the cells exhibiting showing tartaric acid- resistant acid phosphatase activity (TRAP activity), which is a specific marker for osteoclasts, were selectively stained using LEUKOCYTE ACID PHOSPHATASE with leukocyte acid phosphatase kit (Sigma Co., Ltd.). As a result of microscopic observation, TRAP positive cells were not detected in the wells in which COS-7 cells transfected with pcDL-SR α 296 were fixed. In contrast using a microscope, 45 ± 18 (average \pm standard deviation, n = 3) TRAP positive cells

were observed in the wells in which COS-7 cells pOBM291-transfected COS-7 cells were fixed, while no cells showing TRAP activity were detected in the wells in which pcDL-SR α 296-transfected with pOBM291COS-7 cells were fixed.

~~Moreover~~Furthermore, it was also confirmed that calcitonin bound to ~~thesesaid~~ TRAP positive cells. ~~Based on these findings~~Thereby, it has ~~been proven~~was revealed that OBM ~~hashad an activity to promote osteoclast formation-indueing activity.~~

<[Example 17]>

Osteoclast formation-inducing activity**Osteoclastogenesis Promoting Activities of Trx-OBM and sSecretedory-formtype OBM**

Mouse spleen cells were suspended in α -MEM containing 10^{-8} M of the active-form of vitamin D₃, 10^{-7} M dexamethasone, and 10% bovine fetal bovine-serum atin a concentration of 2×10^6 cells/ml. ~~The, and~~ 350 μ l of this suspension was added to each well of a 24 -well plate in the amount of 350 μ l per well. Each well was then charged with 350 μ l of athe solution-prepared (40 ng/ml) obtained by diluting the purified Trx-OBM with the above-mentioned culture medium-(40 ng/ml), 350 μ l of solution prepared~~obtained~~ by 10-fold-diluting the conditioned medium which was produced by culturing of 293-EBNA cells, ~~in-~~ (which were transduced by pCEP sOBM or pCEP4 were transfected,cultured in IMDM-10% FCS; with the above-mentioned culture medium,) so that the concentraion was 1/10, or 350 μ l ~~only~~ of the above-mentioned culture medium. ~~The alone~~ was added to each well. Then, a Millicell® PCF (Meillipore Co., Ltd.) was set on each well, to ~~whichand~~ 600 μ l of ST2-cells ~~which was suspended~~ 4×10^4 cells/ml suspension of ST2 cell in the above-mentioned culture medium (4×10^4 /ml)-were added to the Millicell® PCF. After the cells were cultured for ~~six~~6 days, the Millicell® PCF was removed. ~~The, and the~~ plate was washed once with PBS-and. When the cells were fixed with acetone-ethanol solution (50: 50) for ~~one~~1 minute. ~~Then, only~~ the cells exhibiting ~~theshowing~~ tartaric acid resistant acidie phosphatase activity (TRAP activity) were ~~selectively stained usingwith~~ LEUKOCYTE ACID PHOSPHATASE kit (Sigma Co., Ltd.). ~~The result of microscopie~~Through observation revealed that no cells exhibiting the TRAP activity were detected in the wells to which no Trx-OBM was addedunder a microscope, whereas $106 \pm \pm 21$ (average $\pm \pm$ standard deviation, n = 3) TRAP- positive cells were observed in the wells to ~~whichwhen~~ Trx-OBM was added.

while no cells showing TRAP activity were detected in the wells when not added. Similarly, while no cells exhibiting TRAP activity were detected in the wells to which conditioned medium of 293-EBNA transfected with pCEP4 had been added, 120 ± 31 (average \pm standard deviation, $n = 3$) TRAP positive cells were observed in the wells to which the conditioned medium of 293-EBNA transfected with pCEPsOBM had been added, while no cells showing TRAP activity were detected in the wells when not added. Moreover, it was also confirmed that calcitonin binds to these TRAP positive cells. These results have proven that Trx-OBM and secretory-type OBM exhibit an activity to promote osteoclast formation-inducing activity.

<[Example 18]>

Identity of the Protein OBM Expressed by the cDNA of the Present Invention and the Natural-Type OCIF-Binding Protein of the Present Invention

(1) —

(1) Preparation of rabbit anti-OBM of Anti-OBM Rabbit Polyclonal Antibody
Three male Japanese white rabbits (weight: 2.5- to 3.0 kg, supplied by Kitayama Labesth Co., Ltd.) were immunized subjected to hypodermic immunization with the purified OBM (thioredoxin-OBM fusion protein) produced according to the method in Examples 14(6) and 14(7) by subcutaneously injecting 1 ml/dose of emulsion prepared by mixing 200 μ g/ml of the purified OBM (thioredoxin-OBM fusion protein), which was obtained in accordance with equal volume the methods described in Examples 14-(6) and 14-(7), with 200 μ g/ml of Freund's complete adjuvant (Difco Difco Co., Ltd.). The immunization was carried out 6 times, once a in total with one-week-Ten days after interval each, and all the blood was collected from the rabbits on the 10th day counted from the last immunization, the rabbits were exsanguinated. An antibody was purified from the fractionated serum as follows in the following manner. Ammonium sulfate was added to the That is, the antiserum which was diluted two-fold with PBS to be 1/2 concentraion, and ammonium sulfate was added thereto so that the final concentration of was 40% (w/v%). After being allowed Then, the antiserum was left to stand at 4°C for one hour at 4°C, the mixture

was and centrifuged at 8,000 X g for 20 minutes at 8000 x g to obtain a precipitate. Thereafter, the precipitate was collected and dissolved in a small aliquot of PBS, and then dialyzed against PBS at 4°C, and loaded to. The resulting solution was charged onto a Protein G-Sepharose® column (manufactured by Pharmacia Co., Ltd.). After washing the column with PBS, the adsorbed immunoglobulin G adsorbed was eluted with 0.1 M glycine-HCl hydrochloric acid buffer solution (pH 3.0). The eluate, and the pH thereof was immediately neutralized adjusted to be neutral with 1.5 M Tris-HCl hydrochloric acid buffer (pH 8.7). After dialyzing the eluted protein fractions fraction was dialyzed against PBS, the absorbance at 280 nm was measured to determine the protein and its concentration was determined ($E^{1\%}_{1\text{cm}}$ 13.5). Anti-Horseradish peroxidase-labeled anti-OBM antibody labeled with horseradish peroxidase was prepared using a maleimide-activated peroxidase kit with Maleimide Activated Peroxidase Kit (Pierce Co., Ltd.) as follows. That is, 80 µg of N-succinimide-S-acetylthioacetate acetylthioacetic acid was added to 1 mg of the purified antibody and reacted allowed to react at room temperature for 30 minutes. Five mg of hydroxylamine was added thereto for deacetylation, and then the resulting mixture to deacetylate the antibody. The modified antibody was fractionated by using a polyacrylamide desalting column. The protein fractions were mixed with 1 mg of maleimide-activated peroxidase and reacted for one hour allowed to react at room temperature to obtain for 1 hour, and then the enzyme-labeled antibody was obtained.

(2) — Capability of rabbit anti-OBM polyclonal antibody to inhibit specific binding
Inhibition of Specific Binding of the protein (OBM)-expressed Protein Expressed by the cDNA of the present invention Present Invention (OBM) or the natural type protein Natural-Type Protein of the present invention with to OCIF by Anti-OBM Rabbit Polyclonal Antibody

Purified 2 µg/ml of purified OBM (thioredoxin-OBM fusion protein) obtained according to in accordance with the methods described in the Examples 15-(6) and 15-(7) and the 2 µg/ml of natural -type purified OCIF-binding protein of the Example 2-(4) were dissolved respectively in 0.1 M sodium carbonate buffer to a concentration of 2 µg/ml hydrogencarbonate, respectively. An aliquot 100 µl of each solution was added 100 µl per to each well respectively to of a 96-well immunoplate (manufactured by Nunc

Co.), Ltd. ~~The plate was allowed~~) and then left to stand at 4°C overnight at 4°C. 200 µl of 50% ~~Block Ace~~BLOCKACE was added to each well and the plate was allowed ~~left~~ to stand at room temperature for ~~one~~1 hour. After washing each well ~~wells~~ three times with PBS containing 0.1% Polysorbate 20 (P20-PBS), ~~100~~200 µg/ml of rabbit anti-OBM rabbit antibody solution which was dissolved in 25% ~~Block Ace~~prepared BLOCKACE ~~diluted~~ with P20-PBS to a concentration, and 100 µl of ~~200 µg/ml~~the antibody solution or ~~100 µl of 25% Block Ace (containing no BLOCKACE without antibody)~~ was added to each well, ~~followed by incubation and incubated~~ at 37°C for ~~one~~1 hour. Each well was washed ~~After washing wells~~ three times with P20-PBS and charged with, 100 µl/well of a medium for the binding test solution ~~experiment~~ (P20-PBS containing 0.2% calf bovine serum albumin, 20 mM Hepes; and 0.1 mg/ml ~~h~~Heparin) to which ~~containing~~ 20 ng/ml of the ¹²⁵I-labeled OCIF described in the Example 8-(3) was added ~~thereto~~.

~~Alternatively~~Furthermore, each well was charged with 100 µl/well of another medium for the binding test solution ~~experiment~~ containing 8 µg/ml of unlabeled OCIF in addition to 20 ng/ml of the ¹²⁵I-labeled OCIF ~~was added to other wells~~. After incubating these immunoplates at 37°C for ~~one~~1 hour, the ~~each wells were~~ was washed ~~six times~~ with P20-PBS ~~six times~~. The amount of ¹²⁵I in each well was measured ~~by~~with a gamma counter. The results are shown in ~~Figure~~Fig. 17. As shown in ~~the figure~~, both the ~~purified~~Fig. 17, neither OBM expressed ~~using~~obtained by expressing the cDNA of the present invention and the protein that specifically binds ~~subsequently purifying or the~~ natural -type OCIF ~~specifically binding protein of the present invention do not bind to,~~ which specifically binds OCIF, bound the ¹²⁵I-labeled OCIF at all, when they were treated with the rabbit anti-OBM polyclonal antibody, whereas both proteins bound ~~rabbit antibody~~. On the other hand, it was confirmed that both proteins bound to the ¹²⁵I-labeled OCIF when ~~untreated~~ not treated with the antibody. The binding of both proteins to ¹²⁵I-labeled ~~said antibody~~. Furthermore, it was also revealed that ~~bindings of both proteins to~~ OCIF ~~was confirmed to be clearly were~~ specific, because ~~these binding since the bindings~~ are significantly inhibited by the addition of a 400-fold ~~higer~~ concentration of unlabelled OCIF (8 µg/ml). ~~Based on~~From the above results described above, it was revealed that the rabbit anti-OBM ~~rabbit~~ polyclonal antibody recognizes ~~both the OBM~~ which ~~is~~was the protein expressed ~~using~~by the cDNA of the present invention and the

natural-type OCIF- binding protein of the present invention, and ~~it inhibits the~~inhibited specific binding of ~~these~~both proteins ~~with~~to OCIF.

<[Example 19]>

Cloning of hHuman OBM cDNA

(1) Preparation of mMouse OBM pPrimer

~~The~~For screening of human OBM cDNA, a mouse OBM primers prepared
~~according to~~primer prepared in accordance with the method of the ~~Examples (above~~
~~Example, OBM #3 and OBM#8)~~described above, were used for screening of human
~~OBM cDNA #8 were used. The~~Sequences sequences thereof are shown in the Sequence
~~Table, Sequence~~SEQ ID No. NO: 9 and No. 6, respectively. SEQ ID NO: 6.

(2) Acquisition of Human OBM cDNA Fragments by PCR

~~(2)——Isolation of~~A human OBM cDNA fragment by PCR~~Human OBM cDNA~~
~~fragments were~~was obtained by PCR method using the mouse OBM cDNA primers
~~prepared in (1) above and Human Lymph Node Marathon ready cDNA (Clontech Co.,~~
~~Ltd.) which was a human lymph node derived cDNA library as a template~~and using
~~the mouse OBM cDNA primer prepared in the above (1).~~

The following are the conditions used for PCR~~were shown as follows:~~

10- x <u>X</u> EX Taq buffer (Takara Shuzo Co.,
22.0 µl
2.5 mM dNTP 1.6 µl
cDNA solution+1.0 µl
EX Taq (Takara Shuzo Co., Ltd.) 0.2 µl
Distilled w <u>W</u> ater14.8 µl
40 µM p <u>P</u> rimers OBM #3
0.2 µl
40 µM p <u>P</u> rimers OBM #8
0.2 µl

~~These~~After the above solutions were mixed together in a microfuge tube and pre-
incubated, PCR was conducted under the following conditions. A pretreatment was

~~carried out at 95°C for 2 minutes, followed by 40 cycles of a three-stage then the cycle reaction consisting of reactions at 95°C for 30 seconds, at 57°C for 30 seconds; and at 72°C for 2.5 minutes. After the reaction was repeated 40 times, and the solution was incubated at 72°C for an approx. 5 minutes at 72°C and a portion. A subfraction of the solution was subjected to reaction product and run through agarose by electrophoresis on an agarose gel. A detected an approximate 690 bp DNA fragment (about 690 bp) amplified by the with the above mouse OBM cDNA primers described above was detected.~~

(3) Purification of the human Human OBM cDNA fragment amplified Amplified by PCR and dDetermination of the nucleotide sequence Nucleotide Sequence

~~The human OBM cDNA fragments obtained in the Example 19-(2) was were separated by agarose gel electrophoresis on an agarose gel and further then purified using by use of a QIAEX® gel extraction kit (Qiagen QIAGEN Co., Ltd.). PCR was again performed using the By use of the purified human OBM cDNA fragments as a template and the templates, PCR was conducted again by use of the above mouse OBM cDNA primers described above, primer so as to produce prepare a large quantity amount of the human OBM cDNA fragment. The DNA fragment was fragments which were then purified by use of the QIAEX® gel extraction kit in the same manner as above. The nucleotide sequence of the purified human OBM cDNA fragment was determined using by use of a Taq Dye Deoxy Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.) using OBM #3 or and OBM #8 (Sequence SEQ ID No. NO: 9 and No. 6 SEQ ID NO: 6, respectively) as a primer primers. When compared with the Comparing the nucleotide sequence of the human OBM cDNA fragment with the corresponding are a part of the mouse OBM cDNA, the nucleotide sequence of the human OBM cDNA fragment showed 80.7% they share a homology with that of the mouse OBM cDNA 80.7%.~~

(4) Screening of a full length human for Full Length Human OBM cDNA by hybridization using the human Hybridization With Human OBM cDNA fragment (about Fragments With a Length of About 690 bp) as a probe Probes

~~A full length The human OBM cDNA was screened using the human OBM cDNA fragment (fragments, with a length of about 690 bp) that was, purified in the Example 19-(3) and were labeled with [α - 32 P] dCTP using by use of a~~

MegaprimeMEGA PRIME DNA L_aabeling kit (Amersham Co., Ltd.), and full length human OBM cDNA was screened. As an object to be screened, a Human Lymph Node 5' -STRETCH PLUS cDNA library (Clontech Co., the U.S.Ltd.A, USA) was screened using the DNA probe used. According to the manufacturer'sIn accordance with a protocol issued by the company, after *Escherichia coli* C600 Hfl was infected with the recombinant phage at 37°C for 15 minutes at 37°C. The infected, the *Escherichia coli* was added to an LB agar medium (1% trypton, 0.5% yeast extract, 1% NaCl, 0.7% agar) which was heated at 45°C. The LB agar was and poured onto an LB agar medium plate containing 1.5% agar. After overnight incubationculturing at 37°C, HyBond-
HYBOND® N™ (Amersham Co., Ltd.) was placed to brought into intimate contact with the plate on whichhaving plaques were produced and storedformed thereon for about 3 minutes. According to a conventional methodThen, theis filter was treated withsubjected to an alkaline solutiondenaturation treatment in accordance with a commonly used method, neutralized, and dippedimmersed in a 2×SSC× SSC solution. The DNA was then immobilized ontofixed on the filter using theby UV CROSSLINKER (Stratagene Co., Ltd.). The resulting-obtained filter-was dipped into was immersed in a Rapid-hyb buffer (Amersham Co.), Ltd.-After pretreatment) and pretreated at 65°C for 15 minutes at 65°C, the. Thereafter, the filter was placed in Rapid-hyb transferred into the above buffer containing the above heat- denatured human OBM cDNA fragments (about 690 bp, 5×10^5 cpm/ml) described above. Afterand allowed to hybridize at 65°C overnight hybridization at 65°C. After the reaction, the filter was washed with 2 × SSC, 1 × SSC, and 0.1 × SSC, each 0.1%-SDS-containing 0.1% SDS2X SSC once, with 1X SSC once and with 0.1X SSC once in this order respectivelyturn at 65°C for 15 minutes at 65°C. SeveralThe obtained positive clones obtained-were further purified by repeatingsscreened two more times so as to purify the screening twiceclones. A clone possessing an insert (having about 2.2 kb) of insert was selected from the purified clonesout of these and was used in the following experiments. This purified phage was named λ -hOBM.
AboutFrom the purified λ hOBM, about 10 µg of DNA was obtained from the purified λ -hOBM usingin accordance with a protocol of a QIAGEN® Lambda kit (QiagenQIAGEN Co., Ltd.) according to the manufacturer's protocol. TheAfter this DNA was digestedcleaved with a restriction enzyme SalI and subjected to electrophoresis

~~on an agarose gel to separate the~~ Sall, about 2.2 kb of hOBM insert cDNA (about 2.2 kb) ~~was separated by agarose electrophoresis. This~~ DNA fragment, purified ~~using the~~ by use of a QIAEX® gel extraction kit (Qiagen QIAGEN Co., Ltd.), was ~~digested~~ cleaved with restriction enzyme ~~SaH~~ Sall in advance and then inserted into dephosphorylated plasmid pUC19 (MBI Co., Ltd.) ~~which was previously digested with a restriction enzyme Sall and dephosphorylated, using by use of a DNA ligation kit ver. 2 (Takara Shuzo Co., Ltd.).~~ *Escherichia coli* DH 5-~~α~~ (Gibco BRL Co., Ltd.) was transformed ~~with by use of~~ the pUC19 containing ~~the resulting~~ obtained DNA fragment. The ~~resulting~~ obtained transformant was named pUC19hOBM. ~~The~~ After proliferating the transformant ~~was grown and pUC19hOBM in which the, about 2.2 kb of human -OBM cDNA (about 2.2 kb) was -inserted and plasmids were purified by thereform in accordance with a conventional commonly used method.~~

(5) Determination of nucleotide sequence the Nucleotide Sequence of cDNA
Encoding the entire amino acid sequence Full Length Amino Acid Sequence of hHuman
OBM

The nucleotide sequence of the ~~resulting~~ human OBM cDNA obtained in Example 19 ~~(4)~~ was determined ~~using the~~ by use of a Taq Dye DeoxyDideoxy Terminator Cycle Sequencing FS kit (Paerkin Elmer Co., Ltd.). Specifically That is, the nucleotide sequence of the inserted fragment was determined ~~using by use of~~ pUC19hOBM as a template. ~~As primers, primers for the determination of the nucleotide sequence of the inserted fragment DNA in pUC19hOBM, M13 Primer M3 and 3, M13 Primer RV (manufactured by Takara Shuzo Co TAKARA SHUZO CO., LTD.), and a synthetic primer, human OBM #8,8 designed based on the nucleotide sequence of the human OBM cDNA fragment (about 690 bp) were used.~~ The as primers for determining the nucleotide sequence of the inserted fragment DNA of pUC19. The sequences of the primers used, M13 Primer M3 and M13 Primer RV, are shown in SEQ ID NO: 4 and SEQ ID NO: 5, respectively shown as the Sequence ID No. 4 and No. 5. The amino acid sequence of human OBM dedueedestimated from the nucleotide sequence of the human OBM cDNA is shown in the Sequence Table as Sequence SEQ ID No. 11. The NO: 11, and the nucleotide sequence of the human OBM cDNA is shown as in Sequence SEQ ID No. NO: 12.

The obtained plasmid containing the human OBM cDNA and the obtained *Escherichia coli* which was transformed by the pUC19hOBM, which is the plasmid containing the resulting human OBM cDNA, was were deposited in with the National Institute of Bioscience and Human Technology, of the Agency of Industrial Science and Technology, on August 13, 1997 as of the Ministry of International Trade and Industry with the deposition No. number FERM BP-6058.6058 on August 13, 1997.

[Example 20]

Radioiodination ¹²⁵I Labeling of OCIF with ¹²⁵I and a Quantitative analysis

Determination of ¹²⁵I-labeled OCIF by ELISA

OCIF was ¹²⁵I-labeled in accordance with ¹²⁵I using the IODO-GEN Iodogen method. Twenty ~~20~~ μl of 2.5 mg/ml IODO-GEN Iodogen-chloroform solution was transferred to a 1.5 ml Eppendorf tube, and the chloroform was evaporated at 40°C; thereby providing a tube so as to prepare an Iodogen-coated with IODO-GEN tube. ~~The~~ After the tube was washed ~~three times~~ with 400 μl of 0.5 M sodium phosphate buffer solution (Na-Pi; pH 7.0) three times, followed by the addition of 5 μl of 0.5 M Na-Pi (with a pH of 7.0). To this tube was added. Immediately after 1.3 μl (18.5 MBq) of Na-¹²⁵I solution (Amersham Co., Ltd., NEZ-033H), immediately followed by was added to the addition of tube, 10 μl of 1 mg/ml OCIF solution (monomer type or dimer type) was added. The mixture resulting solution was mixed in by means of a vortex mixer and allowed left to stand at room temperature for 30 seconds. This solution was transferred to a tube ~~to which containing~~ 80 μl of 0.5 M Na-Pi (pH 7.0) solution containing (pH 7.0), which contained 10 mg/ml potassium iodide and 5 μl of a phosphate buffered saline solution containing 5% bovine serum albumin (BSA-PBS) were previously added, and mixed. The solution was ~~mixed, applied~~ added to a spin column (1 ml, G-25 Sephadex® fine, ~~manufactured by Pharmacia Co., Ltd.) which was equilibrated with BSA-PBS in advance, and centrifuged at 2,000 rpm for 5 minutes at 2,000 rpm.~~ Four ~~After hundred~~ 400 μl of BSA-PBS was added to the a fractions eluted from the column. After mixing and the fraction was mixed, 2 μl of the solution each fraction was used to measure sampled, and the radioactivity of the sample was measured by means of a gamma counter. The radiochemical purity of the prepared ¹²⁵I-labeled OCIF solution obtained above was measured determined by counting measuring the

radioactivity of ~~fractions~~ a fraction precipitated by addition 10% trichloroacetic acid (TCA).

The OCIF biological activity of the ^{125}I -labeled OCIF was measured ~~according to~~ their accordance with a method described in WO 96/26217. The ~~Further, the~~ concentration of the ^{125}I -labeled OCIF was measured ~~using the~~ by ELISA method as follows in the following manner. Specifically That is, 100 μl of 50 mM NaHCO_3 (pH 9.6) in which, having 2 $\mu\text{g}/\text{ml}$ of rabbit anti-OCIF polyclonal antibody described in the WO 96/26217 was dissolved to a concentration of 2 $\mu\text{g}/\text{ml}$ therein, was added to each well of a 96-well immunoplate (MaxiSorpTM, manufactured by Nunc Co.) in the amount of 100 μl per well. After these wells were allowed, Ltd., MaxiSorp) and left to stand at 4°C overnight at 4°C. After this solution was removed. Then the wells were charged with a mixed aqueous ~~discarded, 200 μl of mix-solution of Bloek AceTM~~ BLOCKACE (Snow Brand Milk Products Co., Ltd.) and a phosphate buffered saline solution (mixing ratio = 25:75) (B-PBS) in the amount of 200 μl was added to each well. The plate was and then allowed left to stand for two hours at room temperature for 2 hours. After the solution was removed ~~discarded, the each wells were was~~ washed three times with a phosphate buffered saline solution containing 0.01% Polysorbate 80 (P-PBS) three times. Next ~~Thereafter, 100 μl of B-PBS containing a~~ ^{125}I -labeled OCIF sample or the OCIF reference standard OCIF was added in the amount of 100 μl to each well. The plate was then allowed and left to stand for two hours at room temperature for 2 hours. After the solution was removed discarded, each well was washed six times with 200 μl of P-PBS six times. A Then, 100 μl of diluted solution prepared by diluting of peroxidase-labeled rabbit anti-OCIF rabbit polyclonal antibody within B-PBS was added in the amount of 100 μl to each well. The plate was allowed and left to stand for two hours at room temperature for 2 hours. After the solution was removed discarded, the each wells were was washed six times with 200 μl of P-PBS six times. Then, a 100 μl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added in the amount of 100 μl to each well. After being allowed and then left to stand at room temperature for 2- to 3 minutes. Thereafter, 100 μl of a termination solution (Stopping Reagent, (Scytek Co., Ltd.) was added to each well. Absorbance The absorbance of each well at 450 nm was measured at 450 nm using by means of a microplate reader. The

concentration of the ^{125}I -labeled OCIF was determined with from a calibration curve prepared using by use of the OCIF reference standard-OCIF.

<[Example 21]>

Expression of ~~the protein encoded~~ Protein Encoded by ~~the~~ cDNA of the ~~p~~Present ~~i~~Invention

(1) Construction of hOBM eExpression vVector for aAnimal eCells

pUChOBM was ~~digested~~cleaved with restriction enzyme SaHSall, and a DNA fragment (about 2.2 kb) ~~were~~ DNA fragments were purified by 1% agarose gel electrophoresis on an 1% agarose gel. The ends of the DNA fragments were blunted using a and blunt-ended with DNA bBlunting kKit (Takara Shuzo Co., Ltd.) (blunted hOBMeDNA the resulting DNA fragment with smoothed terminals is called "smoothed hOBM cDNA fragment"). Expression plasmid pcDL-SR α -296 (Molecular and Cellular Biology, Vol. 8, pp. 466- to 472 (1988)) was ~~digested~~cleaved with a restriction enzyme EcoRI, blunted with and blunt-ended with the blunting kit and ligated with the blunted hOBM. The resulted expression plasmid was bound to the smoothed hOBM cDNA fragment using by use of a DNA ligation kit ver. 2. Using the ligation reaction solution, *Escherichia coli* DH- α was transformed with the ligation reaction. A plasmid in. From the resulting obtained ampicillin -resistant transformant was subjected to digestion with restriction enzyme to analyze the DNA restriction map and determine the DNA sequence. As a result, a strainclone, having a phOBM plasmid in which hOBM cDNA is inserted in the samewith forward direction of for transcription as that direction of SR- α promoter α promoter, was selected by analysis of DNA map obtained by restriction enzyme cleavage and determination of DNA sequences. The microorganism obtained strainclone was named DH5- α /phOBM.

(2) Expression of hHuman OBM in COS-7 eCells

Escherichia coli, DH5- α /phOBM, was cultured and the plasmid phOBM was purified using Qiafilter with QIA® Filter Plasmid Midi kKit (Qiagen QIAGEN Co., Ltd.). The phOBM was transfected using Lipofectamine into COS-7 cells in the each wells of a 6- well plate by use of lipofectamine, and the cells were cultured for two days in DMEM containing 10% fetal bovine serum for 2 days. The culture medium was replaced with cysteine-/methionine-free DMEM (manufactured by Dainippon Seiyaku Pharmaceutical

Co., Ltd.) to which containing 5% dialyzed fetal bovine serum was added (88 μ l/well). The, and the cells were incubated cultured for another 15 minutes. Then, followed by addition of 14 μ l of Express Protein Labeling Mix (NEN Co., Ltd., 10 mCi/ml) was added. After four the cells were cultured for 4 hours incubation, 200 μ l of DMEM containing 10% fetal bovine serum was added to each well. The, and the cells were cultured for one 1 hour and. After the cells were washed twice with PBS. Then twice, 0.5 ml of a-TSA buffer (10 mM Tris-HCl (pH 8.0) containing 0.14 M NaCl and 0.025% NaN₃, pH 8.0) containing 1% Triton X-100, 1% bovine hemoglobin, 10 μ g/ml leupeptin, 0.2 TIU/ml aprotinin, and 1 mM PMSF was added to each well, and the mixtures cells were allowed left to stand for one hour on ice for 1 hour. The After the cells were mixed crushed by pipetting and, the resulting lysate was centrifuged at 4°C and 3,000 \times g for 10 minutes at so 4°C, as to obtain supernatants a supernatant. Two hundred 200 μ l of a dilution buffer (TSA buffer containing 0.1% Triton X-100, 0.1% bovine hemoglobin, 10 μ g/ml leupeptin, 0.2 TIU/ml aprotinin, and 1 mM PMSF) was added to 100 μ l of the supernatant from each well. The, and the resulting mixtures were agitated at 4°C for one hours supernatant was shaken together with Protein A Sepharose® (50 μ l) and at 4°C for 1 hour. Thereafter, the solution was centrifuged at 4°C, 1,500 \times g for one 1 minute at so 4°C, as to collect supernatants a supernatant. Thereby, thereby removing the a protein which non-specifically adsorbed binding the Protein A Sepharose® was removed. OCIF (1 μ g) was added to the supernatants supernatant, and the mixtures were agitated resulting supernatant was shaken at 4°C for one 1 hour at so 4°C as to bind human-OBM and OCIF together. Then, rabbit anti-OCIF rabbit polyclonal antibody (50 μ g) was added, followed by agitation and the resulting solution was shaken at 4°C for one 1 hour. Then, Protein A Sepharose® (10 μ l) was added to the resulting solution, followed by agitation and the solution was then shaken at 4°C for an additional 1 hour. The mixtures thus obtained were resolution was centrifuged at 4°C, 1,500 \times g for 1 minute at 1,500 \times g at 4°C so as to collect precipitates a precipitated fraction. The precipitates were precipitate resulting from the centrifugation was washed twice with a the dilution buffer twice, twice with a bovine hemoglobin- free dilution buffer twice, once with a TSA buffer once, and once with 50 mM Tris-HCl (pH 6.5) once. After addition washing,

of an SDS buffer containing 10% β -mercaptoethanol (0.125 M Tris-HCl, 4% dodecyl sodium dodecylsulfate, 20% glycerol, 0.002% Bromophenol Blue, pH 6.8), the mixture containing 10% β -mercaptoethanol was added to the precipitate. The precipitate was heated at 100°C for 5 minutes at 100°C, and it was subjected to SDS-PAGE (12.5% polyacrylamide gel, Daiichi Pure Chemical Kagaku Co., Ltd.). The gel was fixed and dried according to in accordance with a conventional commonly used method. After isotope, and the signals of isotopes from the fixed gel were enhanced amplified using by Amplify™ (Amersham Co.), the dried Ltd.). The fixed gel was subjected exposed to autoradiography at -80°C using Bio-Max BioMax® MR film (Kodak Co., Ltd.) at -80°C. The results are shown in Figure 18, which shows Fig. 8. As a result, it was revealed that the molecular weight of the protein encoded by the cDNA of the present invention is was about 40,000.

<[Example 22]>

Binding of the protein encoded Protein Encoded by the cDNA of the pPresent iInvention and to OCIF

PhOBM, which was purified in In the same manner as in the Example 21-(2), the purified phOBM was transfected into COS-7 cells in each well of a 24- well plate using Lipofectamine. After by the use of lipofectamine, and the cells were cultured for 2 to or 3 days. Then, the cells were washed with serum-free DMEM. Two hundred, and 200 μ l of a culture medium for at the binding test medium assay (serum-free DMEM to which containing 0.2% bovine serum albumin, 20 mM Hepes buffer solution, 0.1 mg/ml heparin, and 0.2% NaN_3 were added), containing 20 ng/ml of ^{125}I - labeled OCIF was added to the some wells. To the In addition, to other wells, 200 μ l of culture medium for the binding test medium the medium for binding assay, containing 8 μ g/ml of unlabeled OCIF in addition to 20 ng/ml of the ^{125}I - labeled OCIF, was added. After incubation for one hour at 37°C in a so as to conduct following experiments. After culture in a CO_2 incubator (5% CO_2), the cells were washed twice with 500 μ l of a phosphate buffered saline solution containing 0.1 mg/ml of heparin. Then, 500 μ l of 0.1 N NaOH solution was added to each well and the plate was allowed at 37°C for 1 hour, the cells were washed twice with 500 μ l of phosphate buffered saline containing 0.1 mg/ml heparin. After washing, 500 μ l of 0.1 N NaOH solution was added to each well, and the wells

were then left to stand for 10 minutes at room temperature to dissolve the for 10 minutes so as to dissolve the cells. The radioactivity amount of ^{125}I in the wells was measured by a gamma counter. As a result, as shown in Figure 19, in each well was measured by means of a gamma counter. As a result, it was confirmed that the ^{125}I -labeled OCIF binds only to the cells transfected with phOBM. Moreover, as shown in Fig. 19. Further, it was also confirmed that the binding was significantly inhibited by adding 400-fold excess unlabelled OCIF (8 $\mu\text{g/ml}$). Based on the results described above, the protein, human OBM encoded by the cDNA in the phOBM was confirmed to specifically bind to OCIF on the surface of was significantly inhibited by addition of a 400-fold concentration of unlabeled OCIF (8 $\mu\text{g/ml}$). From these results, it was revealed that a human OBM protein, coded for by a cDNA on phOBM, specifically bound to OCIF on the surface of a COS-7 cells.

<.

[Example-23>

23]

Crosslinking of ^{125}I -labeled OCIF and Experiment of ^{125}I Labeled OCIF to Protein Encoded by the cDNA of the Present Invention

To further analyze the characteristics of the protein encoded by the cDNA of the present invention, crosslinking of ^{125}I labeled monomer type OCIF with the protein encoded by the cDNA of the present invention Crosslinking of ^{125}I -labeled monomer type OCIF and the protein encoded by the cDNA of the present invention was carried out to further investigate the characteristics of the protein encoded by the cDNA of the present invention. After constructing expression vector phOBM and transfecting into COS-7 cells according to the method used in the was conducted. That is, after expression vectors phOBM were prepared and transfected into COS-7 cells in accordance with the methods described in Examples 21-(1) and 21(2), 200 μl of binding test medium the medium for the binding assay containing the ^{125}I -labeled OCIF (25 ng/ml) was added to some wells. In addition, the medium for the binding assay, containing unlabeled OCIF of a 400-fold concentration in addition to the ^{125}I labeled OCIF (25 ng/ml) described above, was added. The binding test medium to which unlabeled OCIF was added at a

400-fold concentration in addition to ^{125}I -labeled OCIF was used for the other wells. After cultured for one hour at 37°C in a other wells. The cells were cultured in a CO₂ incubator (5% CO₂), the cells were washed twice with 500 µl of phosphate buffered saline containing 0.1 mg/ml heparin. Five hundred µl of phosphate buffered saline in which at 37°C for 1 hour, and the cells were washed twice with 500 µl of phosphate buffered saline containing 0.1 mg/ml of heparin. To these cells, 500 µl of phosphate buffered saline containing 100 µg/ml of a crosslinking agent (DSS:-(dDisuccinimidyl suberate, manufactured by Pierce Co., Ltd.) was dissolved was added to, and the cells, followed by incubation incubated at 0°C for 10 minutes at for 0°C reaction. The After the cells in these wells were washed twice with 1 ml of ice-cold phosphate buffered saline. After an addition of cooled to 0°C, 100 µl of 20 mM Hepes buffer solution containing 1% Triton X-100 (Wako Pure Chemicals Co. Chemical Industries, Ltd.), 2 mM PMSF (Pphenylmethylsulfonyl fluoride, Sigma Co., Ltd.), 10 µM Pepstatin (Wako Pure Chemicals Co. Industries, Ltd.), 10 µM leupeptin (Wako Pure Chemicals Co. Chemical Industries, Ltd.), 10 µM antipain (Wako Pure Chemicals Co. Chemical Industries, Ltd.) and 2 mM EDTA (Wako Pure Chemicals Co. Industries, Ltd.) were added to these cells, and the wells were allowed left to stand at room temperature for 30 minutes at room temperature minute so as to dissolve lyse the cells. These After 15 µl of these samples (15 µl aliquots) were treated with SDS under reducing nonreducing conditions according to in accordance with a conventional commonly used method and, the samples were subjected to electrophoresis with a gel for SDS- electrophoresis using (4- to 20% polyacrylamide gradient gel-(Daiichi Pure Chemical Kagaku Co., Ltd.). After the electrophoresis, the gel was dried and subjected exposed to autoradiography for 24 hours at -80°C using BioMax® MS film Film (Kodak Co., Ltd.) with BioMax® MS Intensifying Amplifying Screen (Kodak Co. Co., Ltd.) and BioMax MS sensitization screen (Kodak Co.) at -80°C for 24 hours. The film subjected to autoradiography was exposed films were developed according to in accordance with a conventional commonly used method. As a result, a protein band of having a molecular weight in the range of about 90,000-110,000, shown in Figure 20, to 110,000 was detected as shown in Fig. 20 by crosslinking of between ^{125}I -labeled monomer type OCIF and the protein encoded by the cDNA of the present invention.

<[Example 24]>

Expression of sSecretedory-form-Type hHuman OBM

(1) Construction of sSecretedory-formType hHuman OBM eExpressiøng pPlasmid

A PCR reaction was carried out usingby use of human OBM SF (Sequence Table, SequenceSEQ ID No:NO: 13) and mouse OBM #8 (Sequence Table, SequenceSEQ ID No:NO: 6) as primers and pUC19hOBM as a template. After the product was purified by agarose gel electrophoresis ~~on an agarose gel~~, the product was digested, it was cleaved with restriction enzymes SP4HSpI and HindIII, and furtherthen purified by agarose gel electrophoresis ~~on an agarose gel~~ so as to obtain a purified fragment (0.27 kb) fragment. Human OBMA fragment of hOBM cDNA which was partially digested with cleaved at only one site of restriction enzyme DraI by partial cleavage of human OBM cDNA therewith, and DNA fragments digested with DraI at one site were purified by agarose gel electrophoresis ~~on an agarose gel~~. The, and the purified fragment was further digestedcleaved with a restriction enzyme HindIII. The 0.53 kb of DraI/HindIII fragment was purified by agarose gel electrophoresis ~~on an agarose gel~~. The, and the purified fragment was ligated withand the 0.27 kb Sp4HSpI/HindIII fragment derived from fragment (0.27 kb) of the PCR described above using ligation kit ver. 2 (Takara Shuzo Co.) PCR product together with HindIIHan SpI/EcoRIV fragment (5.2 kb) of pSec TagA (InvitrogenInvitrogen Co.), Ltd.) were subjected to ligation by use of a ligation kit ver. 2 (TAKARA SHUZO CO., LTD.), and Escherichia coli DH5-α wasα were transformed usingby use of the reaction product of ligation. Plasmids were purified by alkali SDS method from the resultingobtained ampicillin -resistant transformants and digested withclone by alkaline SDS method and cleaved by restriction enzymes so as to select a plasmid containinghaving 0.27 kb and 0.53 kb- of fragments as insertsinserted in pSec TagA. Thise plasmid was confirmedsubjected to have a sequence encoding the secreted human OBM by sequencing using Tag dyedeoxyterminator cycle sequencing by use of a Taq Dideoxy Terminator Cycle Sequencing FS kKit (Perkin Elmer Co., Ltd.), thereby confirming that the plasmid had sequences encoding secretory-type human OBM. TheAfter the plasmid was digestedcleaved withby restriction enzymes NheII and XhoI to prepareXhoI, a fragment (0.8 kb) corresponding to the secretedsecretory-type human OBM cDNA was collected by agarose gel electrophoresis on an agarose gel. This

fragment was inserted into the NheI and XhoI fragment (10.4 kb) of an expression vector pCEP4 (Invitrogen Co., Ltd.) using by use of the ligation kit, and *Escherichia coli* DH5- α were transformed using by use of the reaction product of the ligation. Plasmids were purified by alkali-SDS method from the resulting obtained ampicillin - resistant transformants clones by alkaline SDS method and digested cleaved with by restriction enzymes so as to select an *Escherichia coli* clone having the a secretory-type human OBM expression plasmid for secreted form human OBM (pCEPshOBM) with a target structure. The *Escherichia coli* containing clone having the pCEPshOBM was cultured, and the pCEPshOBM was purified using Qiafilter plasmid midi kit by use of QIA® Filter Plasmid Midi Kit (Qiagen QIAGEN Co., Ltd.).

(2) Expression of sSecretedory-form Type OBM

293-EBNA cells were suspended in IMDM containing 10% FCS (IMDM-10% FCS), added into a seeded in a collagen-coated 24- well plate coated with collagen (manufactured by Sumitomo Bakelite Co., Ltd.) in a cell density an amount of 2×10^5 cells/2 ml/well, and cultured overnight. The To the cells were transfected with, 1 μ g of pCEPshOBM or pCEP4 using was transfected by use of 4 μ l of Lipofectamine (Gibco Co., Ltd. -After), and the cells were cultured for two another 2 days in 0.5 ml of a serum-free IMDM or IMDM-10% FCS, the culture supernatants were collected thereby collecting a conditioned medium. Expression of the secreted secretory-type human OBM in the culture conditioned supernatant medium was detected as follows confirmed in the following manner. Sodium bicarbonate That is, sodium hydrogen carbonate was added to the culture conditioned supernatants medium to a final concentration of 0.1 M and the mixtures were added to a 96- well plate. The plate was allowed left to stand at 4°C overnight at 4°C, thereby and immobilizing the human OBM in the culture supernatants on the conditioned medium was solid-phased in a 96- well plate. The plate was blocked using Block Ace™ BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution four-fold diluted 4 times with PBS (B-PBS) was added to each well and allowed the plate was left to stand for two hours at room temperature for 2 hours to cause blocking. After adding 3-100 ng/ml of OCIF which was diluted with B-PBS was added to each well, the plate was allowed wells and left to stand at 37°C for two 2 hours at 37°C, followed by wash. After the plate was washed with PBS containing 0.05% Polysorbate 20 (P-PBS).

Then, 100 μ l of a peroxidase- labeled-rabbit anti-OCIF polyclonal-antibody described in WO 96/26217 which was diluted with B-PBS was added to each well. After allowing and left to stand at 37°C for two hours at 37°C, the wells were. After each well was washed six times with P-PBS. Then six times, 100 μ l of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added in the amount of 100 μ l per to each well and the mixture was allowed then left to stand at room temperature for about 10 minutes. The reaction was terminated by the addition of Thereafter, 100 μ l of termination solution (Stopping Reagent, (Scytek Co., Ltd.) was added to each well. Absorbance The absorbance of each well at 450 nm for each well was measured by means of a microplate reader. The results are shown in Figure 21, which indicates that the absorbance at 450 nm increased according to the concentration of the added OCIF in Fig. 21. In the plate in which having the solid-phased conditioned medium of the cells transfected with the pCEPshOBM was immobilized, absorption at 450 nm increased depending on the concentration of the OCIF added. On Meanwhile, in the case where the conditioned medium of the other hand, no increase in absorbance was seen in the wells in which the conditioned medium of the cells cells transfected with only with the vector pCEP4 was immobilized-solid-phased, no increase in absorption was seen. Further, Figure Fig. 22 shows the results of an experiment wherein in which the proportion of the conditioned medium used for immobilizationsolid phasing was changedvaried within a range of 5- to 90% in the presence ofand a consertaint concentration of OCIF (50 ng/ml) was added. The absorbanceIn the plate having the solid-phased conditioned medium of the cells transfected with the pCEPshOBM, absorption at 450 nm increased according to thealong with an increase in the proportion of the conditioned medium in the plate wherein the conditioned medium of the cells transfected with pCEPshOBM was immobilizedadded. Meanwhile, whereas no such increase in absorbance was seen in the plate wherein having the solid-phased conditioned medium of the cells transfected with the vector pCEP44, no increase in absorption was immobilizedobserved. From these results, it was confirmed that secretedory-formtype human OBM iswas producedexpressed in the conditioned medium of the cells transfected with pCBPshOBMthe pCEPshOBM.

<[Example 25]>

Expression of ~~Thioredoxin-h~~Human OBM ~~Fusion p~~Protein (Trx-hOBM)

(1) Construction of a thioredoxinThioredoxin-hHuman OBM ~~Fusion p~~Protein (Trx-hOBM) ~~e~~Expression ~~v~~Vector

~~Ten~~10 μ l of 10X ExTaq buffer (Takara Shuzo Co., Ltd.), 8 μ l of 10 mM dNTP (Takara Shuzo Co., Ltd.), 77.5 μ l of sterilized distilled water, 2 μ l of ~~an~~pUC19hOBM aqueous solution of ~~p~~pUC19hOBM (10 ng/ μ l), 1 μ l of primer; mouse OBM #3 (SEQ ID NO: 9) (100 pmol/ μ l, Sequence Table, Sequence ID No. 9), 1 μ l of primer; hOBM SalR2 (SEQ ID NO: 14) (100 pmol/ μ l, Sequence Table, Sequence ID No. 14), and 0.5 μ l of ExTaq (5-u/ μ l) (Takara Shuzo Co., Ltd.) were mixed and reacted (PCR) together in a ~~micro-centrifuge~~microcentrifuge tube so as to cause a PCR reaction. After the reaction at~~consisting of~~ 95°C for 5 minutes, at 50°C for ~~one~~1 second, at 55°C for ~~one~~1 minute, at 74°C for ~~one~~1 second, and at 72°C for 5 minutes, at~~the~~ cycle reaction consisting of a reaction at 96°C for ~~one~~1 minute, at 50°C for ~~one~~1 second, at 55°C for ~~one~~1 minute, at 74°C for ~~one~~1 second, and at 72°C for 3 minutes, was repeated 25 times. From the total reaction mixtureAn approximately 750 bp DNA fragment (~~750 bp~~) was purified. ~~The~~ from the whole amount of reaction solution. After the purified DNA fragment (whole) was digestedcleaved with restriction enzymes SalI (Takara Shuzo CoTAKARA SHUZO CO., LTD.) and BspHI (New England Bilabs CoNEW ENGLAND BILABS CO., LTD.), and subjected to electrophoresis on a 1% agarose gel electrophoretic migration was carried out so as to obtain purifiedpurify an approximately 320 bp DNA fragment (Fragment 1, about 320 bp). The fragment was dissolved 1) and dissolve the fragment in 20 μ l of sterilized distilled water. In the same manner, Similarly, an approximately 450 bp DNA fragment (Ffragment 2, about 450 bp) obtained by digesting 2) which is a cleaved product of 4 μ g of pUC19hOBM withdescribed in Example 19-(3) by a restriction enzymes BamHI, and BspHI (Takara Shuzo CoTAKARA SHUZO CO., LTD.) and about 3.6 kb of DNA fragment (Fragment 3, about 3.6 kbfragment 3), obtained by digesting which is a cleaved product of 2 μ g of pTrXFus (InVytrogen Co., Ltd.) withby a restriction enzymes BamHI, and SalI (Takara Shuzo CoTAKARA SHUZO CO., LTD.) were respectively purified and then dissolved in 20 μ l of sterilized distilled water. The QIAEXHTo purify the DNA fragments, a QIAEXR

II gel extraction kit was used for purification of the DNA fragments. Fragments 1- Fragment 1, 2 and 3 were ligated combined by incubating at 16°C for 2.5 hours using use of a DNA ligation kit ver. 2 (Takara Shuzo Co TAKARA SHUZO CO., LTD.) by keeping them at 16°C for 2.5 hours. Using the ligation reaction, Escherichia coli GI724 strain (Invitrogen Co., Ltd.) was transformed according to the using the ligation reaction solution, in accordance with a method described in the Instruction Manual of an instruction manual attached to a ThioFusion Expression System (Invitrogen Co., Ltd.). A microorganism strain with From the obtained ampicillin-resistant transformants, a clone, having a plasmid in which the an hOBM cDNA fragment is fused in framewas bound to a thioredoxin gene in the same reading frame, was selected from the resulting ampicillin resistant transformants by analysis of DNA restriction map mapping obtained by digestion with restriction enzyme cleavage and by determination of DNA sequences. The microorganism strain thus obtained strain was named GI724/pTrxhOBM-25.

(2) Expression of Trx-hOBM OBM in Escherichia coli

A GI724/pTrxhOBM strain and a GI724 containing strain transformed with pTrxFus (GI724/pTrxFus) were respectively cultured six hours with shaking at 3037°C for 6 hours in 2 ml of RMG-Amp medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 2% casamino acid, 1% glycerol, 1 mM MgCl₂, 100 µg/ml ampicillin, pH 7.4). The broth (0.5 ml) of the culture suspension was added to 50 ml of Induction medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 0.2% casamino acid, 0.5% glucose, 1 mM MgCl₂, 100 µg/ml ampicillin, pH 7.4) and cultured with shaking at 30°C. When OD_{600nm} reached about 0.5, L-tryptophan was added so as to achieve a final concentration of 0.1 mg/ml, followed by culturing with when the value at OD_{600 nm} became about 0.5, and the cells were further shaking-cultured at 30°C for an additional another 6 hours. The cculture brothsuspension was centrifuged at 30003,000 xX g so as to collect the cells, which wereand then the collected cell was suspended in 12.5 ml of PBS. The suspension was subjected to an ultrasonic generator (Ultrasonics Co., Ltd.) so as to disruptcrush the cells. Then-disrupt cells-were the sample was centrifuged at 70007,000 xX g for 30 minutes so as to obtain a supernatant liquid as a collect a soluble protein fraction as a supernatant. Ten10 µl of this soluble proteinthe solution fraction was subjected to SDS-PAGE (10% polyacrylamide-(10%)

electrophoresis under reducing conditions. As a result, as shown in Figure Fig. 23, a protein band with having a molecular weight of 40,000 about 40,000, which was could not be seen in the soluble protein fraction of GI724/pTrxFus, was detected in the soluble protein fraction of GI724/pTrxhOBMpTrxOBM. Accordingly From the above results, it was confirmed that a thioredoxin-human OBM fusion protein (Trx-hOBM) of thioredoxin and human OBM) was expressed in the *Escherichia coli* clone.

(3) Binding Ability of Trx-hOBM *Escherichia coli* (3) — Binding capability of Trx-hOBM to OCIF

Binding of the expressed Trx-hOBM to OCIF It was confirmed according to by the following experiment that the expressed Trx-hOBM bound to OCIF. Anti That is, 100 μ l of anti-thioredoxin antibody (Invitrogen Co., Ltd.) which was diluted 5000 fold to be 1/5,000 with 10 mM sodium hydrogen carbonate aqueous solution was added to each well of a 96- well immunoplate (Nunc Co., Ltd.), in and the amount of 100 μ l per well. After being allowed plate was left to stand at 4°C overnight at 4°C. After the liquid solution in the each well cell was discarded. Two, hundred 200 μ l of a solution prepared obtained by diluting Block Ace™ BLOCKACE (Snow Brand Milk Products Co., Ltd.) two fold to be 1/2 with PBS (BA-PBS) was added to each well. After being allowed, and then the plate was left to stand for one hour at room temperature, for 1 hour. After the wells were solution was discarded, each well was washed with P-PBS three times with P-PBS. The 100 μ l of the GI724/pTrxOBM-derived soluble protein fractions originating from fraction solution diluted stepwise with BA-PBS, and 100 μ l of the above-described GI724/pTrxhOBM or GI724/pTrxFus, each-derived soluble protein fraction solution diluted stepwise with BA-PBS in various concentrations, were added to each well in and the amount of 100 μ l. After being allowed plate was left to stand for two hours at room temperature, for 2 hours. After each well was washed with P-PBS three times with P-PBS and charged with, 100 μ l of OCIF (100 ng/ml) which was diluted with BA-PBS. After being allowed, was added to each well and the plate was left to stand for two hours at room temperature, for 2 hours. After each well was washed with P-PBS three times with P-PBS and charged with, 100 μ l of peroxidase-labeled anti-OCIF antibody (described in WO 96/26217) which was 26217, diluted to be 1/2,000 fold with BA-PBS.

After being allowed, was added to each well, and the plate was left to stand for two hours at room temperature, for 2 hours. After each well was washed six times with P-PBS and charged with six times, 100 μ l of TMB solution. After being allowed was added to each well, and then the plate was left to stand at room temperature for about 10 minutes at room temperature. Thereafter, each well was charged with 100 μ l of termination solution (Stopping Reagent). Absorbance was added to each well. The absorbance of each well at 450 nm was measured by means of a microplate reader. The results are shown in Figure Fig. 24. There was no difference in the absorbance was observed between absorbance resulted in the sample with presence and absence of the soluble protein fraction originating from GI724/pTrxFus-added thereto-derived soluble protein fraction solution and, while with the sample without the addition of this GI724/pTrxhOBM-derived soluble protein fraction. On the other hand solution, the absorbance increased depending on an increase in the samples to which the soluble protein fraction originating from GI724/pTrxhOBM was added in proportion to the concentration of the GI724/pTrxOBM derived soluble protein fraction solution. The Further, Fig. 25 shows the results of the other an experiment wherein in which the dilution rate of the soluble protein fraction solution to be added was maintained kept constant (1% concentration) while adding and OCIF (0-100 ng/ml) diluted stepwise with BA-PBS in different concentrations (0-100 ng/ml) are shown in Figure 25. was added. It can be seen that For the GI724/pTrxFus-derived soluble protein fraction solution, absorbance remained low at any concentrations regardless of the concentration of OCIF in samples using a soluble protein fraction originating from GI724/pTrxFus, whereas while for the GI724/pTrxhOBM-derived soluble protein fraction solution, absorbance increased in proportion to the OCIF concentration in the samples to which the soluble protein fraction originating from GI724/pTrxhOBM was added concentration-dependent manner. Based on these results, it was confirmed from this result that Trx-hOBM which is produced from GI724/pTrxhOBM has a capability of binding had an ability to bind OCIF.

(4) Large Scale cultivation Culture of *Escherichia coli* which produces Producing Trx-hOBM

GI724/pTrxhOBM cells ~~were~~ spread on an RMG-Amp agar medium (0.6% Na_2HPO_4 , 0.3% KH_2PO_4 , 0.05% NaCl, 0.1% NH_4Cl , 2% casamino acid, 1.5% agar, pH 7.4) ~~using~~ with a platinum transfer loop. The cells ~~were~~ loop and cultured at 30°C overnight at 30°C . The cultured cells were suspended in 10 ml of Induction medium. ~~The~~, and every 5 ml of the suspension was added (5 ml ~~for~~ to each) ~~to~~ of two 2-L Erlenmeyer conical flasks of 2L volume containing 500 ml of Induction medium, and the flasks were shaking-cultured at 30°C with shaking. When the $\text{OD}_{600\text{nm}}$ reached about 0.5, L-tryptophan was added so as to achieve a final concentration of 0.1 mg/ml. ~~Culturing~~ with when absorbance at $\text{OD}_{600\text{nm}}$ became about 0.5, and the shaking culture at 30°C was continued for ~~six~~ another 6 hours at 30°C . The culture broth suspension was centrifuged at $3,000 \times g$ for 20 minutes at $3000 \times g$ so as to collect cells and the collected cells, which were then suspended in 160 ml of PBS. The suspension was subjected to an ultrasonic generator ~~ultrasocination~~ (Ultrasonics Co., Ltd.) so as to ~~disrupt~~ crush the cells. The supernatant liquid, and the cell lysate was then centrifuged at $7,000 \times g$ for 30 minutes at $7000 \times g$ so as to ~~obtain~~ collect a soluble protein fraction as a supernatant.

(5) Preparation of OCIF-immobilized Affinity Column

Two 2 g of TSKgel AF-Tsresyl Toyopal ~~TOYOPAL~~ 650 (Tosoh Corp ~~TOSO CO., LTD.~~) and 40 ml of 1.0 M potassium phosphate buffer (pH 7.5) containing 35.0 mg of recombinant OCIF, which was prepared according to the by a method described in WO 96/26217, were mixed. ~~The mixture was together and~~ gently shaken at 4°C overnight at 4°C so as to effect cause a coupling reaction. ~~The reaction mixture was centrifuged to remove the supernatant.~~ To inactivate excessive active residues ~~residue~~, after a supernatant was removed by centrifugation, 40 ml of 0.1 M Tris-HCl buffer (pH 7.5) was added to the precipitated carrier, and the mixture was gently shaken at room temperature for one hour. ~~The carrier in a column was washed with~~ After 0.1 M glycine-HCl buffer (pH 3.3) containing 0.01% Polysorbate 80 and 0.2 M NaCl and (pH 3.3) and a 0.1 M sodium citrate buffer (pH 2.0) containing 0.01% Polysorbate 80 and 0.2 M NaCl. ~~The carrier (pH 2.0) were passed through a column (in which the obtained gel was packed) so as to wash it,~~ the column was equilibrated by charging washed twice with 10 mM sodium phosphate buffer (pH 7.4) containing 0.01% Polysorbate 80 ~~80~~ (pH 7.4) so as to equilibrate it.

(6) Purification of Trx-hOBM using OCIF-immobilized Affinity Column

~~Unless otherwise indicated, purification~~ Purification of Trx-hOBM was carried out at 4°C ~~unless otherwise stated~~. ~~The~~ After the above-mentioned OCIF-immobilized affinity carrier (10 ml) and the ~~above-mentioned~~ soluble protein fraction ~~solution~~ (120 ml) ~~prepared~~ ~~described~~ in Example 25-(4) were mixed. ~~The together, the mixture was~~ gently shaken at 4°C overnight ~~at 4°C~~ in four 50 ml centrifuge tubes ~~using by use of a~~ rotor. ~~An Econo-column™~~ The carrier in the mixture was filled an EconoColumn (internal diameter: 1.5 cm, length: 15 cm, ~~manufactured by BioRad~~ Bio-Rad Co., Ltd.) ~~was filled with the carrier in the mixture~~. The column was charged with 300 ml of PBS containing 0.01% Polysorbate 80, 100 ml of 10 mM sodium-phosphate buffer (pH 7.0) containing 0.01% Polysorbate 80 and 2.0 M NaCl (pH 7.0), and 100 ml of 0.1 M glycine-HCl buffer (pH 3.3) containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH 3.3) ~~were~~ passed through the column, in that order ~~turn, so as to wash the column~~. Next ~~Then,~~ proteins adsorbed in the column were eluted with 0.1 M sodium citrate buffer (pH 2.0) containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH 2.0) ~~was passed through the column so as to elute proteins adsorbed to the column~~. ~~The eluate was collected in 5 ml portions~~ eluates were fractionated. ~~Each fraction thus collected was immediately neutralized with addition of~~ To the fractions, 10% volume of 2 M Tris-buffer solution (pH 8.0) ~~was added so as to immediately neutralize the fractions~~. ~~Presence~~ The presence or absence of Trx-hOBM in the eluted ~~fraction~~ ~~each fraction of the eluate~~ was determined ~~according to~~ ~~examined in accordance with~~ the method ~~previously described in~~ Example 25-(3) ~~(the binding capability to OCIF)~~. The ~~fractions~~ Fractions containing Trx-hOBM were collected and purified further.

(7) Purification of Trx-hOBM by gGel Filtration

About 25 ml of ~~the~~ Trx-hOBM fractions ~~obtained~~ ~~described~~ in Example 25-(6) was concentrated ~~using a centrifuge~~ to about 0.5 ml by ~~centrifuging using use of a~~ Centriplus R10 and a Centricon R10 (Amicon Co., Ltd.). ~~This~~ The concentrated sample was ~~applied~~ ~~subjected~~ to a Superose R12 HR 10/30 column (1.0 x 30 cm, Pharmacia Co., Ltd.) ~~previously equilibrated in advance~~ with PBS containing 0.01% Polysorbate 80. ~~For the separation,~~ The column was developed at a flow rate of 0.25 ml/min by using PBS containing 0.01% Polysorbate 80 ~~was used as a mobile phase at a flow rate of 0.25~~

~~ml/min. The eluate so as to fractionate every 0.25 ml of eluates from the column was collected in 0.25 ml portions. The Trx-hOBMOBM in the thus collected fractions was detected by the same method as previously described in the Example 25-(3) and SDS-PAGE. Fractions containing purified Trx-hOBM were collected and so as to measure the protein concentration of Trx-hOBM was determined OBM. The measurement of the protein concentration was carried out measured with DC-protein assay kit (Bio-Rad Co., Ltd.) using bovine serum albumin as a reference standard substance using DC Protein assay kit (BioRad Co.).~~

<[Example 26]>

Osteoclast formation inducing activity of hOBM

Osteoclastogenesis Inducing Activity of OBM

phOBM and pcDL-SR- α 296 were respectively transfected into COS-7 cells using Lipofectamine by use of lipofectamine (Gibco Co., Ltd.), respectively. ~~The~~ After the cells were cultured for one day in DMEM containing 10% FCS for 1 day, they were trypsinized and seeded in a 24-well plate, ~~plated on~~ in which glass cover slips (15 mm round shape, manufactured by Matsunami Co., Ltd.) in 24-well plates were seated, at a concentration of 5×10^4 cells per/well, and then cultured for ~~2~~ another two days. The culture plate was washed ~~once~~ with PBS. The cells were fixed with ~~once and then~~ PBS containing 1% paraformaldehyde was added, and the cells were incubated at room temperature for 8 minutes so as to fix the cells on the glass cover slips. ~~The~~ After the plate ~~on which the~~ with fixed cells were attached was washed 6 times with PBS, then six times, 700 μ l of mouse spleen cells suspended at 1×10^6 /ml in α -MEM (containing 10^{-8} M active form activated vitamin D₃, 10^{-7} M dexamethasone, and 10% fetal bovine serum) in an amount of 1×10^6 cells/ml were added to each well. ~~Millicell~~ MILLICELL® PCF (Millipore Co., Ltd.) was set ~~in on~~ each well, and a 700 suspension μ l of ST2 cells, suspended in the above-mentioned culture medium (in a concentration of 4×10^4 cells/ml) were added, 700 μ l per well, into to the ~~Millicell~~ MILLICELL® PCF followed by incubation and cultured at 37°C for 6 days. After the culture ~~that~~, the ~~Millicell~~ MILLICELL® PCF was removed, and the plate was washed ~~once~~ with PBS, and once. Then, the cells were fixed with for a minute by an acetone-ethanol solution (50:50) for one minute. ~~Then, the~~ and cells, exhibiting having tartaric acid-resistant acid

phosphatase activity (TRAP activity), which is a specific marker for osteoclast, were selectively stained using LEUKOCYTE ACID PHOSPHATASE by use of a leukocyte acid phosphatase kit (Sigma Co., Ltd.). As a result of microscopic observation, cells having TRAP-positive activity were not detected in the wells in which COS-7 cells transfected with the pcDL-SR $\alpha 296$ were fixed. In contrast, $\alpha 296$, while 65 ± 18 ($n = 3$, average \pm standard deviation, $n=3$) of TRAP positive cells were observed in the wells in which COS-7 cells transfected with phOBM were fixed. Moreover, expression of Further, it was also confirmed that these TRAP positive cells expressed calcitonin receptor was confirmed by receptors, since the fact that cells showed specific binding to ^{125}I -labeled salmon calcitonin (Amersham Co., Ltd.) specifically bound to these TRAP-positive cells. Based on From these findings results, it has been proven was revealed that human OBM, which is the a protein encoded by the cDNA of the present invention, has had an activity to promote osteoclast formation-inducing activity.

<[Example 27]>

Osteoclast formation-inducing activity **Osteoclastogenesis Promoting Activities of Trx-hOBM and sSecretedory-form Type hHuman OBM**

Mouse spleen cells were suspended in α -MEM containing 10^{-8} M active-form activated vitamin D₃, 10^{-7} M dexamethasone, and 10% fetal bovine serum at a concentration of 2×10^6 cells/ml. The, and 350 μl of the suspension was added to each well of a 24 well plate in the amount of 350 μl per well. Each well was then charged with After 350 μl of a solution prepared by diluting purified Trx-hOBM OBM, (40 ng/ml) with the above-mentioned culture medium (40 ng/ml), 350 μl of a solution prepared by 10-fold-diluting a conditioned medium which was produced by culturing obtained when 293-EBNA cells, onto which transduced by pCEPshOBM or pCEP4 were transfected, cultured in a culture medium IMDM-10% FCS, to be 1/10 with the above-mentioned culture medium, or 350 μl only of the above-mentioned culture medium. The Millicell PCF (Millipore alone was added, a MILLICELL® PCF (Millipore Co., Ltd.) was placed set on each well, to which and 600 μl of ST2 cells which were suspended cell suspension in the above-mentioned culture medium (4×10^4 cells/ml) were added to the Millicell® PCF. After the cell were cultured for

six days, the Millicell® PCF was removed. The, and the plate was washed once with PBS and once. Then, after the cells were fixed for 1 minute by an acetone-ethanol solution (50:50) for one minute. Then, the cells exhibiting the activity of having tartaric acid resistant acid phosphatase activity (TRAP activity) were selectively stained using LEUKOCYTE ACID PHOSPHATASE by use of a leukocyte acid phosphatase kit (Sigma Co., Ltd.). The results of microscopic observation revealed that no Using a microscope, cells exhibiting the having TRAP activity were not detected in the wells to which not containing Trx-hOBM was added, whereas while 115 ± 19 ($n = 3$, average \pm standard deviation, $n=3$) of TRAP- positive cells were observed in the wells to which containing Trx-hOBM was added. Similarly, while no cells exhibiting having TRAP activity were not detected in the wells to containing which the conditioned medium of 293pCEP4-EBNA cells transfected with pCEP4 had been added 293-EBNA, while 125 ± 23 ($n = 3$, average \pm standard deviation, $n=3$) of TRAP positive cells were observed in the wells to containing which the conditioned medium of 293pCEPshOBM-EBNA cells transfected with pCEPshOBM had been added 293-EBNA. Moreover Furthermore, expression of calcitonin receptor it was also confirmed by the fact that these TRAP positive cells expressed calcitonin receptors, since the cells showed specific binding to ^{125}I - labeled salmon calcitonin (Amersham Co., Ltd.) specifically binds to. From these TRAP positive cells. These results have proven, it was revealed that Trx-hOBM and secreted form hOBM exhibit secretory-type OBM had an activity to promote osteoclast formation inducing activity.

<[Example 28]>

Preparation of pPolyclonal aAntibody

Mouse sOBM or human sOBM, which was used as an immunogen immunizing antigen, was prepared according to obtained in accordance with the above-mentioned method described above. Especially That is, mouse sOBM cDNA (cDNA (Sequence ID No. 18) encoding which encodes mouse sOBM (Sequence SEQ ID No. NO: 16) which does not have the having no membrane binding region of the mouse OBM due to absence of the site and lacking amino acids from between the N-terminal down to the 72nd end and amino acid 72 of mouse OBM; SEQ ID NO: 18) or human sOBM cDNA (cDNA (Sequence ID No. 19) encoding which encodes human sOBM (Sequence SEQ ID No. NO:

17) ~~which does not have the~~having no membrane binding region of human OBM due to absence of the ~~site and lacking amino acids from region between~~ the N- terminal down to the end and amino acid 71~~st amino acid of human OBM; SEQ ID NO: 19~~)-was ligated, together with a Hind III/EcoRV fragment (5.2 kb) of the ~~a~~ pSec TagA expression vector pSec-TagA (InVytrogen Co., Ltd.)~~including the,~~ containing nucleotide sequence ~~encoding~~coding a signal peptide of κ -chain of immunoglobulin, ~~together with~~and an EcoRI/PmaCI fragment (0.32 kb) of OBM cDNA, ~~using~~were subjected to ligation by use of a ligation kit ver. 2 (Takara Shuzo Co. ~~TAKARA SHUZO CO., LTD.~~). *Escherichia coli* DH5- α ~~was~~are transformed with the reaction product. ~~The plasmids~~Plasmids ~~were purified from the~~ obtained ~~from the resulting~~ ampicillin -resistant strains ~~were purified by the alkali~~clones by alkaline SDS method and ~~digested with and~~cleaved by restriction ~~enzyme~~enzymes so as to select a plasmid ~~with~~having 0.6 K~~b~~kb and 0.32 kb of fragments inserted ~~into~~in pSec TagA. ~~The sequence~~As a result of this~~determining the~~ sequences of the plasmid ~~was identified using the Dye~~deoxyterminator by use of Dye Terminator Cycle Sequencing FS kit (product of Perkin Elmer Co.), Ltd.~~As a result~~, it was confirmed that this ~~plasmid has a sequence~~had sequences encoding mouse or human sOBM. After ~~The~~ plasmid was ~~digested~~cleaved ~~with~~by restriction enzymes ~~NheI/XhoI,~~NheI and XhoI and then a fragment (1.0 kb) corresponding to secretion~~y~~ form-type OBM cDNA was ~~recovered~~collected by agarose gel electrophoresis. This fragment was inserted into an ~~NheI/XhoI~~ fragment (10.4 kb) of the ~~an~~ expression vector pCEP4 (InVytrogen Co., Ltd.) ~~using~~by use of a ligation kit~~-, and~~ *Escherichia coli* DH5- α ~~was~~are transformed ~~using~~by use of the reaction product. Plasmids were purified by the alkali-SDS ~~from the resulting~~obtained ampicillin -resistant strains~~-. Analyzing these plasmids by digesting with a~~clones by an alkaline SDS method and cleaved by restriction enzyme,~~enzymes and analyzed so as to select a~~ *Escherichia coli* ~~possessing~~clone having a secretion-type~~secretory~~ OBM expression plasmid (pCEP sOBM) ~~having with the~~ objective target structure~~was selected.~~ The *Escherichia coli* ~~strain~~clone having the pCEP sOBM was cultured, and the pCEP sOBM was purified ~~using~~by use of a Qiafilter-plasmid midy kit QIA® Filter Plasmid Midi Kit (Qiagen QIAGEN Co., Ltd.). Next, 293-EBNA cells ~~were~~was suspended in IMDM containing 10% FCS (IMDM-10% FCS) ~~containing 10% FCS and~~ ~~plated~~seeded ~~onto~~in a-24- collagen-coated 24 well plate~~-coated with~~

collagen (product of Sumitomo Bakelite Co., Ltd.) at a cell density in an amount of 2×10^5 cells/2 ml/well. After, and culturing overnight, To the cells were transformed with, 1 μg of pCEP sOBM or pCEP4 using was transfected by use of 4 μl of Lipofectamine (Gibco Co., Ltd.), and further the cells were cultured for two another 2 days in 0.5 ml of serum-free IMDM or IMDM-10% FCS, thereby collecting a conditioned medium. The culture supernatant was recovered. A cell line clones with high productivity of recombinant mouse soluble OBM (msOBM) or human soluble OBM (hsOBM) was were screened as follows in the following manner. Sodium bicarbonate After sodium hydrogen carbonate was added to the culture supernatant which is assumed conditioned medium seemed to contain msOBM or hsOBM to at a final concentration of 0.1 M. One, hundred 100 μl of the culture supernatant conditioned medium was added to each well in of 96- well immunoplates (Nunc Co.) and allowed to stand, Ltd.) and the plate was left to stand at 4°C overnight at 4°C , thereby so as to solid-phase the msOBM or hsOBM in the culture supernatant was immobilized conditioned medium on each well. To each well Then, 200 μl of Block-Ace™ BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution diluted four-fold to 4 times with PBS (B-PBS) was added and the plates were allowed to stand for two hours to each well of the plate and the plate was left to stand at room temperature for 2 hours. After washing each well in the plates three times with PBS (P-PBS) containing 0.1% Polysorbate 20, 20 (P-PBS), 100 μl of each recombinant OCIF (rOCIF) solution (3 diluted stepwise (0-100 ng/ml) diluted serially with PB-PBS was added to each well in and the plates. The plates were allowed plate was left to stand at 37°C for two 2 hours at 37°C . After washing the plates three times with PBS, 100 μl of a peroxidase- labeled anti-OCIF polyclonal antibody (WO 96/26217), diluted with B-PBS, was added to each well. After allowing and the plate was left to stand at 37°C for two 2 hours at 37°C , the wells were washed. After washing six times with P-PBS. Then, 100 μl of TMB solution (TMB Soluble Reagent Agent, High Sensitivity, ScyTek Co., Ltd.) was added to each well in the plates and the plates were allowed left to stand at room temperature for about 10 minutes; subsequently the reaction was terminated by adding. Thereafter, 100 μl of a stopping solution (Stopping Reagent, SeyTek Co.) to each (Scytek Co., Ltd.) was added to each well. The absorbance of each well. Absorbance at 450 nm of each well was measured

using by means of a microplate reader. It was confirmed that in the plate having the solid-phased protein derived from conditioned medium of the clone producing msOBM or hsOBM, the absorbance significantly increased remarkably in proportion to the concentration of the added OCIF. As for the plates in which msOBM or hsOBM in the culture supernatant of the cell line clones producing msOBM or hsOBM was immobilized therein, the cell line that exhibited clones indicating a high rate of increase in the absorbance was selected as a strain with high productivity. Each of the highly producing clones of msOBM or hsOBM selected in the above mentioned manner were mass-cultured on a large scale in any use of IMDM medium containing 5% FCS, using as a medium in 25 T-flasks (T-225). After the cells reached confluency, a 100 ml of fresh culture medium was added to each T-225 flask in the amount of 100 ml per 255 flask and the cells were further cultured for 3- or 4 days, to collect the culture supernatant and then a conditioned medium was collected. These procedures were repeated four times to obtain 10 L of the culture conditioned supernatant medium containing msOBM and 10 liters of the conditioned medium containing hsOBM were obtained. Purified msOBM (About 10 mg) or hsOBM (of purified msOBM and about 12 mg) of purified hsOBM, which shows homogeneous band were uniform (molecular weight: 32 kDa) on terms of SDS-polyacrylamide gel-electrophoresis, were obtained from by carrying out purification on the culture supernatant by means of above-obtained conditioned medium with affinity chromatography using an OCIF-OCIF-immobilized column and gel filtration chromatography according to in accordance with the method described in Examples 25-(6) and 25(7). The thus-obtained purified preparations were used as an antigen for immunization. Each protein-antigen was dissolved in phosphate buffered saline (PBS) at a concentration of 200 µg/ml and emulsified then the solution was mixed with an equivalent volume amount of Freund's complete adjuvant so as to be emulsified. One ml of the each emulsion was subcutaneously administered to each of three Japanese white rabbits at intervals of about once every one week so as to immunize the rabbits. A booster injection was given and when the antibody titer was measured, and when the antibody titer

reached a peak maximum, a booster was carried out. ~~Whole~~ 10 days after the booster, all blood was collected ~~10 days thereafter~~ from all the rabbits. The ~~serum~~ Antiserum was diluted to ~~two-fold times~~ with a binding buffer for pProtein A sepharose Sepharose® chromatography (BioRad Bio-Rad Co., Ltd.) and ~~applied then added~~ to a pProtein A column equilibrated with the ~~same above~~ buffer. After ~~washing~~ the column ~~extensively~~ was efficiently washed with the ~~same above~~ buffer, the ~~an~~ anti-sOBM antibody adsorbed to the column was eluted ~~with by~~ an elution buffer (BioRad Bio-Rad Co., Ltd.) or 0.1 M glycine-HCl buffer, (pH ~~3.0-2.9~~ to 3.0). ~~To~~ In order to immediately neutralize the ~~antibody-containing~~ eluate immediately, the ~~eluate eluted~~ solution was fractionated ~~using by use of~~ a test tubes containing a small amount of 1.0 M Tris-HCl (pH 8.0). The ~~antibody~~ eluate was dialyzed ~~against in~~ PBS at 4°C overnight ~~at 4°C~~. The ~~antibody~~ ~~content~~ amount of protein in the antibody solution was measured ~~by in~~ accordance with the Lowry method using bovine IgG as a standard ~~protein~~. Thus, ~~about 10 mg of the~~ purified immunoglobulin (IgG) containing the polyclonal antibody of the present invention ~~was~~ obtained in an amount of about 10 mg per 1 ml of rabbit antiserum ~~was obtained~~.

<[Example 29]>

Measurements of OBM and sOBM by ELISA Using Polyclonal Antibody

Sandwich ELISAs, using the rabbit anti-hsOBM polyclonal antibody ~~A sandwich ELISA was constructed using the rabbit anti-human sOBM polyclonal antibody obtained in Example 28 as the a solid phase antibody and enzyme- as an enzyme labeled antibody, were constructed.~~ Peroxidase ~~As enzyme labeling, peroxidase (POD) -labeled antibody labeling was prepared according to the~~ carried out in accordance with a method of Ishikawa *et al.* (Ishikawa *et al.*; J. Immunoassay, Vol. 4, 209-327, 1983 to 327, 1983). The anti-human sOBM ~~hsOBM~~ polyclonal antibody obtained in the Example 28 was dissolved in a 0.1 M NaHCO₃ ~~to solution at~~ a concentration of 2 ~~µg/ml. One hundred µg/ml, and 100 µl~~ of the resulting solution was added to each well in ~~of~~ 96-well immunoplates (Nunc Co.), ~~which was then allowed, Ltd.) and the plate was left to stand at room temperature overnight.~~ Next ~~Then~~, 200 ~~µl~~ of 50% Block Ace™ BLOCKACE (Snow Brand Milk Products Co., Ltd.) was added to each well, and the plates ~~were~~ ~~allowed was left~~ to stand ~~for one hour at~~ at room temperature ~~for 1 hour.~~ The ~~Each~~ wells ~~were was~~ washed three times with PBS containing 0.1% Ppolysorbate 20 (washing

buffer). Human three times. The purified human OBM, which was expressed according to the method of same manner as in Example 26 and was purified according to the method of same manner as in Example 2. The purified human OBM₂, and the purified human sOBM₁, prepared obtained in example 28 were serially Example 28, was diluted stepwise with the first primary reaction buffer (0.2 M Tris-HCl buffer, pH 7.2, containing 40% Block Ace BLOCKACE and 0.1% Polysorbate 20 polysorbate 20, pH 7.2), respectively, and 100 μ l of the diluted solution was μ l of each diluent were added to each well in. After the plates. The plates were allowed plate was left to stand at room temperature for two 2 hours, and each well was washed three times with the above-mentioned washing buffer three times. Subsequently, 100 μ l of POD- labeled anti-human sOBM polyclonal antibody, diluted 1000-fold 1,000 times with the second secondary reaction buffer (0.1 M Tris-HCl buffer, pH 7.2, containing 25% Block Ace BLOCKACE and 0.1% Polysorbate 20 polysorbate 20, pH 7.2) was added to each well in and the plates. After the plates were allowed plate was left to stand at room temperature for two 2 hours, each well was washed three times with the washing buffer three times. Next, 100 μ l of enzyme substrate solution (TMB, ScyTek Co., Ltd.) was added to each well in the plates, and the plates were allowed was left to stand at room temperature for 10 minutes, followed by the addition of 100 μ l of a reaction termination stopping solution (Stopping reagent, ScyTek Co., Ltd.) was added to each well so as to stop the enzyme reaction. The absorbance at 450 nm of each well was measured using by use of a microplate reader. The results are shown in Figure Fig. 26. The sandwich ELISA, using the rabbit anti-human sOBM polyclonal antibody recognized, almost equally detected both human sOBM (molecular weight; about 32 kDa) and human OBM (molecular weight; about 40 kDa), with a and measurement sensitivity of was about 12.5×10^{-3} pmol/ml (human OBM: about 500 pg/ml, for human sOBM: OBM, about 400 pg/ml for human sOBM). The measurement It was revealed that measurements of mouse sOBM and mouse OBM by ELISA using the rabbit anti-mouse sOBM polyclonal antibody obtained in the Example 28 was able to could be carried out made in the same manner. It was confirmed that an extremely as described above, measurement sensitivity in measuring mouse OBM or mouse sOBM was similar with that in human OBM or human sOBM, and a very small amount of mouse sOBM or mouse

~~OBM can be measured with almost the same sensitivity as described above.~~
could be measured.

~~As mentioned above, the~~

~~As described above, since the present anti-human sOBM polyclonal antibody of the present invention prepared in the Example 28 can equally recognize both the polyclonal antibody obtained in Example 28 recognized both human sOBM and human OBM antigens. Therefore, the antibody was as antigen equally, it was named an anti-human OBM/human OBM/sOBM polyclonal antibody. Meanwhile, since the anti-mouse sOBM polyclonal antibody. Similarly, the anti-mouse antibody obtained in Example 28 recognized both mouse sOBM and mouse OBM as antigen equally, it was named an anti-mouse OBM/sOBM polyclonal antibody prepared in the Example 28 can equally recognize both the mouse sOBM and mouse OBM antigens. This antibody was therefore named anti-mouse OBM/sOBM polyclonal antibody.~~

<Example 30>

Preparation of monoclonal antibody antibody.

[Example 30]

Preparation of Monoclonal Antibody

The purified human sOBM ~~prepared~~obtained in the Example 28 was used as the antigen for immunization an immunizing antigen. The purified human sOBM was dissolved in physiological phosphate buffered saline solution ~~at~~ a concentration of 10 $\mu\text{g/ml}$ and ~~emulsified by mixing with an equivalent volume of Freund's complete adjuvant~~ $\mu\text{g/ml}$. To the prepared human sOBM solution, an equal amount of Freund's complete adjuvant was added so as to emulsify it. The ~~emulsion was intraperitoneally~~Thereafter, 200 μl of the antigen was administered to BALB into the abdominal cavity of each Balb/c micemouse at an interval of one week for a des~~total of 200 μl three times, once a week, so as to immunize the mice. Next~~Then, the equivalent volume of the Freund's complete adjuvant was added to a physiological saline solution containing 5 $\mu\text{g/ml}$ of the human sOBM, an equal amount of Freund's incomplete adjuvant was added so as to fully emulsify it, and 200 μl of the mixture was suffieiently emulsified. This emulsion was injected intraperitoneally to BALB~~administered to each~~

~~of the above Balb/c mice at a dose of 200 μ l, once a one week intervals for a total of four weeks for immunization times so as to further immunize the mice. One~~ After the passage of one week ~~after from~~ the fourth additional immunization, 100 μ l of ~~aphosphate~~ physiological buffered saline solution containing 10 μ g/ml of the human sOBM was intravenously ~~parenterally~~ administered to each of the ~~BALB~~ Balb/c mice as a ~~afor~~ booster. ~~After three days~~ On the 3rd day after the final immunization, the spleen was ~~extracted removed~~, and spleen cells were separated. ~~The spleen cells were and~~ fused with mouse myeloma cells; P3x63-AgAG8.653 ~~according to in accordance with a~~ conventional ~~known~~ method (Koehler, G. and Milstein, C., Nature, 256, 495 (1975)). ~~The suspended fused cells were~~ After completion of the fusion, the cell suspension was cultured ~~for 10 days in an~~ HAT medium containing hypoxanthine, aminopterin, and thymidine for 10 days. After the myeloma cells ~~were dead perished~~ and hybridomas appeared, the HAT-medium was replaced with an HT medium obtained by removing aminopterin-free from the HAT medium, and the cell culture was continued.

<[Example 31]>

Selection of and Cloning of Hybridoma

~~Since the appearance of the hybridoma and cloning~~ Appearance of hybridomas was ~~recognized 10 days after~~ seen on the 10th day from the start of the cell fusion and culturing in Example 30. ~~Monoclonal antibodies~~ 30, a high affinity antibody recognizing the human sOBM with high affinity and hybridomas and hybridoma producing these antibodies were selected ~~according to in the means of~~ the following ~~procedure using the~~ improved solid phase ELISA ~~which is described below. In addition~~ Further, to select the ~~an~~ anti-OBM monoclonal antibody ~~which recognizes~~ recognizing both of human sOBM and mouse sOBM, the mouse sOBM ~~prepared obtained in the~~ Example 27 was used ~~in addition to as well as~~ human sOBM was used as the ~~an~~ antigen ~~for in~~ the solid phase ELISA. The ~~human~~ Human sOBM and mouse sOBM were ~~each~~ respectively was dissolved in a 0.1 M sodium ~~bicarbonate~~ hydrogen carbonate solution at a concentration of 5 μ g/ml. ~~Fifty ml, and 50 μ l~~ of each antigen solution was added to each well ~~in of a~~ 96-well immunoplates (Nunc Co.), Ltd. ~~The plates were allowed~~, and the plate was left to stand at 4°C overnight ~~so as to immobilize attach~~ the antigens. The antigen solution in each well was discarded. ~~Each well was then filled with, and~~ 200 μ l of 50% Blook

~~Ace™~~BLOCKACE (Snow Brand Milk Products Co., Ltd.) ~~and allowed~~was added to
each well. The plate was left to stand at room temperature for one 1 hour so as to cause
blocking. After each well was washed with a phosphate buffered saline solution (PBS-P)
containing 0.1% Ppolysorbate 20, 40 μ l of calfbovine serum (~~Hiclone Inc~~Hyclone Co.,
Ltd.) was added to each well. ~~Subsequently~~Then, 10 μ l of each hybridoma culture
supernatant conditioned medium was added to each well and ~~each the well plate~~was
incubated left to stand under a serum concentration of 80% at room temperature for two 2
hours in the presence of 80% calf serum so as to cause reaction. ~~The~~An object of the solid
phase ELISA in the presence of 80% calf serum is to select a hybridoma which produce
an antibody ~~which can detect~~capable of binding to a very small amount of human sOBM
or mouse sOBM even in a solution containing high concentration the presence of protein
and in the presence of an immunoreaction interfering a serum-derived immune reaction
inhibiting substance derived from serum in high concentration, i.e. that is, to select a
hybridoma ~~which can produce~~producing an antibody with a having high affinity for
human sOBM or mouse sOBM. After completion of the reaction at room temperature for
two 2 hours, the plates werewas washed with PBS-P, and subsequently, 50 μ l of
diluent of peroxidase- labeled anti-mouse IgG (KPL Co CO., LTD.) diluted 5000-fold to
5,000 times with a physiological saline solution containing 25% Block-AceBLOCKACE
was added to each well. After, and the reaction plate was left to stand at room
temperature for two 2 hours, so as to cause a reaction. After the plate was washed with
PBS-P three times with PBS-P. After the addition of, 50 μ l of an enzyme substrate
solution (TMB, ScyTek Co., Ltd.) was added to each well, the reaction was continued
and left to stand at room temperature for five 5 minutes. ~~The enzymatic reaction was~~
~~stopped by the addition of~~Then, 50 μ l of a termination solution (reaction stopping
reagent (Stopping Reagent, ScyTek Co., Ltd.) was added so as to terminate the enzyme
reaction. Hybridomas ~~which produce antibodies recognizing human sOBM or mouse~~
~~sOBM were selected by measuring~~The absorbance at 450 nm of each well using was
measured by use of a microplate reader (Immune Reader IMMUNOREADER
NJ2000™, 2000, Nippon InterMed Co.), Ltd. Hybridomas) so as to select a hybridoma
producing ~~antibodies exhibiting an antibody which recognizes human sOBM or mouse~~
sOBM. The hybridomas showing particularly high absorbance (OD_{450nm}) were selected.

Cloning of these hybridomas and repeatedly cloned 3 to 5 times by a limiting dilution method was repeated 3 so as to 5 times to establish stable established hybridomas producing antibody stably. Hybridomas exhibiting particularly high Out of the obtained hybridomas, hybridomas having higher antibody productivity were selected among the established antibody-producing hybridoma clones.

<[Example 32]>

Production and purification of monoclonal antibody

The antibody-producing hybridomas obtained in the Example 31, i.e. high-affinity antibody-producing that is, the hybridoma producing an antibody which recognizes human sOBM with high affinity and the hybridoma producing the antibody which produces an antibody showing a cross-reactivity to the with mouse sOBM were cultured, respectively, and Each hybridoma was implanted intraperitoneally to BALB in the abdominal cavity of a Balb/c-mice-based mouse which had been given pristane (Aldrich Chemical Co., Ltd.) about a week before, in an amount of 1×10^6 cells per /mouse) to which pristan (Aldrich Co.) was administered one week previously. After about 2- or 3 weeks, accumulated ascites were collected. The was sampled so as to obtain ascites containing the monoclonal antibody, which recognizes antibody recognized human sOBM of the present invention or both the monoclonal antibody recognizing human sOBM and mouse sOBM. Purified monoclonal antibodies were obtained from the ascites using Protein A column (Pharmacia Co., Ltd.) chromatography in the ascites, was purified according to the purification accordance with the method for purifying an anti-OBM/sOBM polyclonal antibodies using a Protein A column antibody described in the Example 28. The purified monoclonal antibody was thus obtained from the ascites by Protein A column chromatography (Pharmacia Co.).

<[Example 33]>

Antigen specificity of monoclonal antibody

Antigenic Specificity of the Monoclonal Antibody

The antigenic specificities of a monoclonal antibody, which specifically recognizes human sOBM, and the of monoclonal antibody, exhibiting having cross-reactivity to both the with human sOBM and mouse sOBM was investigated, were examined using human sOBM, human-intact human OBM having a

membrane binding regionsite, mouse sOBM, and ~~mouse-intact sOBM~~ mouse OBM having a membrane binding regionsite as antigens. ~~More than thirty kinds~~ Although over 30 types of monoclonal antibodies were obtained. ~~The results on several of~~ representative monoclonal antibodies are shown in Table 1. As a result, it was ~~found~~ revealed that most of anti-human sOBM monoclonal antibodies which specifically recognized human sOBM also ~~recognize the human~~ recognized even intact human OBM having a membrane binding region, ~~but~~ site and did not ~~there~~ recognize mouse OBMs sOBM and the ~~mouse-intact mouse OBM~~ mouse OBM which ~~has~~ having a membrane binding regionsite. ~~On the other hand~~

Meanwhile, ~~it was found that only~~ a few monoclonal antibodies recognizing both ~~the of~~ human sOBM and mouse sOBM were also obtained and it was found that these antibodies ~~exhibit~~ had cross-reactivity ~~to both the with~~ human OBM and mouse OBM. These results ~~show~~ indicate that ~~there is a common antigen recognizing sites, namely a common epitopes, in both the human OBM and mouse OBM~~ had a common antigen recognition site, i.e., epitope. ~~Based on the fact that the~~ Since an anti-human sOBM monoclonal antibody prepared ~~using the by use of~~ human sOBM as an ~~immune~~ antigen also equally recognizes human OBM ~~having, which was a membrane binding region~~. ~~Anti-human sOBM intact protein, the monoclonal antibody was named the an~~ anti-human OBM/sOBM monoclonal antibody.

TABLE 1

Antibody	Antigen			
	hsOBM	hoBM	MsOBM	mOBM
H OBM 1	+	+	-	-
H OBM 2	+	+	-	-
H OBM 3	+	+	-	-
H OBM 4	+	+	-	-
H OBM 5	+	+	-	-
H OBM 6	+	+	-	-
H OBM 7	+	+	-	-
H OBM 8	+	+	-	-
H OBM 9	+	+	+	+
H OBM 10	+	+	-	-
H OBM 11	+	+	-	-
H OBM 12	+	+	-	-
H OBM 13	+	+	+	+
H OBM 14	+	+	-	-

Table 1

Antibody	Antigen			
	hsOBM	hOBM	msOBM	mOBM
H-OBM 1	+	+	-	-
H-OBM 2	+	+	-	-
H-OBM 3	+	+	-	-
H-OBM 4	+	+	-	-
H-OBM 5	+	+	-	-
H-OBM 6	+	+	-	-
H-OBM 7	+	+	-	-
H-OBM 8	+	+	-	-
H-OBM 9	+	+	+	+
H-OBM 10	+	+	-	-
H-OBM 11	+	+	-	-
H-OBM 12	+	+	-	-
H-OBM 13	+	+	+	+
H-OBM 14	+	+	-	-

(hsOBM: human soluble OBM, hOBM: human membrane ~~bonding type~~binding OBM, msOBM: mouse soluble OBM, mOBM: ~~humanmouse~~ membrane ~~bonding type~~binding OBM)

<[Example 34]>

Determination of

Tests of eClass and sSubclass of mMonoclonal aAntibody

The class and subclass of the monoclonal antibody of the present invention were determined by the ~~immunoglobulin class and subclass analysis kit~~use of the Immunoglobulin Class/Subclass Analytical Kit (Amersham Co., Ltd.)~~according to the~~. The tests were conducted in accordance with a protocol ~~indicated~~provided in the kit. The results ~~enof~~ representative monoclonal antibodies are shown in Table 2. ~~As shown in Table 2, the~~The majority of anti-human OBM/sOBM monoclonal antibodies ~~were~~had IgG₁, ~~the others were~~and some antibodies having IgG_{2a} ~~and/or~~ IgG_{2b} were also found. ~~Light chains for~~Further, all of the antibodies were κ chain~~had κ chain as a light chain~~.

TABLE 2

Antibody	IgG ₁	IgG _{2a}	IgG _{2b}	IgG ₃	IgA	κ
H-OBM 8	-	+	-	-	-	+
H-OBM 9	+	-	-	-	-	+
H-OBM 10	+	-	-	-	-	+
H-OBM 11	+	-	-	-	-	+
H-OBM 12	-	-	+	-	-	+

H-OBM-13	+	-	-	-	-	+
H-OBM-14	+	-	-	-	-	+

Table 2

Antibody	IgG ₁	IgG _{2a}	IgG _{2b}	IgG ₃	IgA	IgE
H-OBM-8	+	+	+	+	+	+
H-OBM-9	+	+	+	+	+	+
H-OBM-10	+	+	+	+	+	+
H-OBM-11	+	+	+	+	+	+
H-OBM-12	+	+	+	+	+	+
H-OBM-13	+	+	+	+	+	+
H-OBM-14	+	+	+	+	+	+

[Example 35]

Measurement of the dissociation constant (K_d) of monoclonal antibody for the Monoclonal Antibodies

The dissociation constant of the constants for monoclonal antibodies was measured according to in accordance with a known method (Betrand Friguet *et al.*: Journal of Immunological Methods, 77, 305- to 319, 1986). That is, the purified antibody obtained in the Example 32 was diluted at 5 ng/ml with 0.4 M Tris-HCl buffer (a primary buffer, pH 7.4) containing 40% Block Ace BLOCKACE and 0.1% Ppolysorbate 20 to give a concentration of 5 ng/ml. The solution was mixed with (pH 7.4, primary buffer) and an equivalent volume amount of a diluted solution diluent of the purified human soluble OBM (hsOBM) obtained in Example 28 in 28, prepared with the primary buffer with a at stepwise concentration range of from 6.25 ng/ml to 10 µg/ml. The mixture was allowed, was added and the solution was left to stand at 4°C for 15 hours at so 4°C as to bind the hsOBM to the monoclonal antibody to hsOBM. After 15 hours, the an antibody not bound unbound to the hsOBM (10 µg/ml, 100 µl/well) hsOBM was measured using an immobilized by solid phase ELISA with solid-phased hsOBM (10 µg/ml, 100 µl/well) so as to calculate the dissociation constant of the monoclonal antibody to the hsOBM. In addition, Further, the affinity to for msOBM of an antibody, which is a monoclonal antibody for the hsOBM and also exhibits the antibodies, having cross-reactivity to with mouse soluble OBM (msOBM) and hsOBM, was also measured according to the same method except for by using msOBM instead of in place of hsOBM

at the above-mentioned method. Particularly, the hsOBM. Dissociation constant results of particular antibodies, which exhibit had high affinity to for each antigen of the antigens and are were useful for in enzymatic immunoassay and, binding assay and such, are shown in Table 3.

Table 3

Antibody	Subclass	Antigen	Dissociation constant Kd (M)
H-OBM-1	IgG ₁ (t)	hsOBM	$1 \times 10^{-11} < Kd < 1 \times 10^{-10}$
H-OBM-4	IgG ₁ (t)	hsOBM	$1 \times 10^{-11} < Kd < 1 \times 10^{-10}$
H-OBM-9	IgG ₁ (t)	hsOBM	$1 \times 10^{-9} < Kd < 1 \times 10^{-8}$
H-OBM-9	IgG ₁ (t)	msOBM	$1 \times 10^{-9} < Kd < 1 \times 10^{-7}$

Antibody	Subclass	Antigen	Dissociation Constant Kd (M)
H-OBM-1	IgG ₁ (t)	hsOBM	$1 \times 10^{-11} < Kd < 1 \times 10^{-10}$
H-OBM-4	IgG ₁ (t)	hsOBM	$1 \times 10^{-11} < Kd < 1 \times 10^{-10}$
H-OBM-9	IgG ₁ (t)	hsOBM	$1 \times 10^{-9} < Kd < 1 \times 10^{-8}$
H-OBM-9	IgG ₁ (t)	msOBM	$1 \times 10^{-9} < Kd < 1 \times 10^{-7}$

As a result, the dissociation constants (Kd) of it was found that H-OBM 1 and H-OBM 4 which are the were specific antibodies specific to for human soluble OBM (hsOBM) were in showed a dissociation constant on the order of 10^{-11} M, indicating th that they had very high affinity to for hsOBM. On the other hand Meanwhile, the Kd value of the antibody H-OBM 9 which recognizes was an antibody recognizing both the hsOBM and mouse soluble OBM (msOBM) was in on the order of 10^{-8} M with respect to msOBM and in on the order of 10^{-9} M with respect to hsOBM. In addition Further, the dissociation constant of the other regarding H-OBM 13, which was another antibody which recognizes recognizing both antigens shown in the Table 1, i. e. the dissociation constants of H-OBM 13 for each antigen, was the same as that with respect to both antigens were almost identical with those of H-OBM 9, and these since two both antibodies belong to had the same subclass. These findings suggest the, a possibility was suggested that they are were the identical antibodies which recognizes same antibody recognizing the same epitope of each antigen.

<[Example 36]>

Method for Measuring ~~method of human~~ Human OBM and sOBM by ~~s~~Sandwich ELISA ~~u~~Using ~~a~~Anti-~~h~~Human OBM/sOBM ~~m~~Monoclonal ~~a~~Antibodies

A sandwich ~~Sandwich~~ ELISA was constructed ~~using~~ ~~by use of~~ the two types of high affinity monoclonal antibodies obtained in Example 35, i.e., H-OBM 1 and H-OBM 4, respectively ~~as a solid phase antibody and an enzyme- labeled antibody, respectively.~~ Labeling of ~~Maleimide Activated Peroxidase Kit (Pierce Co., Ltd.)~~ was used for labeling the antibody ~~was carried out using a maleimide activated peroxidase kit (Piers Co.).~~ The H-OBM 1 antibody, ~~H-OBM 1,~~ was dissolved in a 0.1 M sodium bicarbonate ~~hydrogen carbonate~~ solution ~~at a concentration of 10 µg/ml, and 100 µl of the resulting~~ solution was added to each well ~~in of~~ 96-well immunoplates (Nunc ~~company~~ Co., Ltd.). ~~After being allowed~~ The plate was left to stand at 4°C overnight ~~at so 4°C as to~~ immobilize ~~attach~~ the antibody;. After the solution in each well was discarded ~~and,~~ 300 µl of 50% Block Ace™ solution ~~BLOCKACE~~ was added to each well, ~~in and~~ the plates. Each well in the plates ~~plate~~ was blocked ~~by allowing~~ left to stand at room temperature for ~~two~~ 2 hours ~~so as to cause blocking.~~ After the blocking, the plates ~~were~~ was washed with phosphate buffered saline containing 0.1% Ppolysorbate 20 (PBS-P). Human-OBM (hOBM) soluble sOBM and human-soluble- OBM (hsOBM) ~~each~~ were respectively ~~diluted with~~ dissolved in 0.4 M Tris-HCl buffer, (pH 7.4, 7.4) containing 40% Block Ace™ ~~BLOCKACE~~ (Snow Brand Milk Products Co., Ltd.) and 0.1% Ppolysorbate 20 (Wako Pure Chemicals Co-Industries, Ltd.) (the first ~~primary~~ reaction buffer) ~~and diluted so as to prepare test samples with various concentrations. These test samples with different~~ 100 µl of each of test sample, prepared at various concentrations, ~~were~~ was added to each well ~~in the amount of 100 µl per well, and reacted~~ the plate was left to the antibody, H-OBM 1 immobilized on each well ~~by incubating~~ stand at room temperature for ~~two~~ 2 hours: ~~so as to cause a reaction.~~ After two hours, the plates ~~were~~ Thereafter, the plate was washed with PBS-P. Next, ~~and~~ 100 µl of a solution of POD- labeled H-OBM 4 antibody ~~in diluted with~~ 0.2 M Tris-HCl buffer, ~~HC~~ (pH 7.4, 7.4) containing 25% Block Ace™ ~~BLOCKACE~~ and 0.1% Ppolysorbate 20 (the second ~~secondary~~ reaction buffer) was added to each well, ~~followed by further incubating.~~ The plate was left to stand at room temperature for ~~two~~ 2 hours ~~so as to cause a reaction.~~ The plates ~~were then~~ After the plate was washed with PBS-P ~~and,~~ 100 µl of an enzyme substrate solution (TMB, ScyTek

Co., Ltd.) was added to each well to start enzyme reaction. The enzyme reaction was terminated by so as to develop color in the addition wells, and 100 μ l of a reaction termination stopping solution (stopping reagent, ScyTek Co., Ltd.) was added to each well so as to stop the enzyme reaction. The absorbance of each well at 450 nm of each well was measured using by use of a microplate reader. The results are shown in Figure Fig. 27.

As a result, it was confirmed/revealed that the sandwich ELISA constructed using by use of the two types of high affinity anti-human OBM/sOBM monoclonal antibodies obtained in Example 35, i.e., H-OBM 1 and H-OBM 4 with high affinity for 4, detected human OBM/ and human sOBM prepared in the Example 35, equally recognizes human OBM and human sOBM, and is able to measure a very small amount of human OBM or human sOBM at a quantitative limit of. The measurement sensitivity thereof was about 1.25×10^{-3} to 2.5×10^{-3} pmol/ml (about 50- to 100 pg/ml for human OBM with having a molecular weight of about 40 kDa, about 40- to 80 pg/ml for human sOBM with having a molecular weight of about 32 kDa), and very small amounts of human OBM and human sOBM could be measured by the ELISA. The hybridomas which produce Hybridomas producing these two types of anti-human OBM/sOBM monoclonal antibodies, H-OBM 1 and H-OBM 44, were named H-OBM1 and H-OBM4, respectively. The Further, a hybridoma producing H-OBM 9, the anti-human OBM/sOBM monoclonal antibody (H-OBM 9) which recognizes/recognized both mouse OBM and mouse sOBM and also has an osteoclast formation-exhibited osteoclastogenesis inhibitory activity, was named H-OBM9. These hybridomas were deposited with the National Institute of Bioscience and Human Technology, of the Agency of Industrial Science and Technology, on November 5, 1993 of the Ministry of International Trade and Industry with Deposit Nos. numbers FERM BP-6264 (H-OBM 1), FERM BP-6265 (H-OBM 4), and FERM BP-6266 (H-OBM 9) on November 5, 1997.

<[Example 37]>

Measurements of mMouse OBM and mMouse sOBM uUsing a Anti-hHuman OBM/sOBM monoclonal antibody which recognizes mouse Monoclonal Antibody Recognizing Mouse OBM and mMouse sOBM

~~A sandwich ELISA was constructed~~Sandwich ELISAs using the anti-human OBM/sOBM monoclonal antibody, H-OBM 9, ~~which recognizes~~recognizing mouse OBM and mouse sOBM ~~and obtained in Examples 33 and 35 as an a solid -phased antibody in, and using the Examples 33 and 35, and the anti-mouse OBM/sOBM polyclonal antibody obtained in Example 28 as an enzyme- labeled antibody-obtained in the example 28-, were constructed.~~ The mouse Mouse OBM and mouse sOBM were respectively mouse sOBM were diluted stepwise with the first primary reaction buffer to give various concentrations in the same manner as in the Example 35 and then measured sOBM according to the method described in the Example 35, and the mouse OBM and mouse sOBM were detected in the same manner as in Example 36. The results are shown in FigureFig. 28. As a result, it was ~~found~~confirmed that the mouse OBM and mouse sOBM ~~can~~could be similarly measured using H-OBM 9 ~~which is the detected equally by use of the anti-human OBM/sOBM monoclonal antibody-recognizing H-OBM 9 which recognized the mouse OBM and mouse sOBM-of the present invention.~~ As shown by ~~in~~ the results of Example 35, this ~~anti-human OBM/sOBM monoclonal~~ the antibody H-OBM 9 has a high dissociation constant relatively with respect to the mouse sOBM; in other words, namely it has a comparatively the antibody had relatively low affinity ~~to~~ for the mouse sOBM. ~~The sensitivity in~~ Thus, the measurement sensitivities of mouse OBM (molecular weight; about 40 kDa) and mouse sOBM (molecular weight; about 32 kDa) by ~~this~~ the above ELISA assay ~~was~~ were about 25×10^{-3} pmol/ml (about 1 ng/ml for mouse OBM ~~and~~, about 0.8 ng/ml for mouse sOBM).

<[Example 38]>

Assay for Osteoclastogenesis- iInhibitory aActivity of aAnti-OBM/sOBM aAntibody

It is known that an osteoclast-like cells (OCL) ~~are~~is induced~~derived~~ by co-culture of mouse spleen cells and ST2 cells (mouse bone marrow- derived ~~stromal~~ cells; interstitial cell) (Endocrinology, 125, 1805-1813, 1805 to 1,813 (1989)). ~~Capability~~ Thus, it was examined whether derivation of the anti- OCL was inhibited by addition of an OBM/ sOBM antibody ~~to inhibit the OCL formation when added to the co-culture system was studied.~~ ~~Because the~~ Since mouse OBM ~~is~~ was expressed in this co-culture system, aantibodies used in this Example were H-OBM 9 and rabbit anti-mouse OBM/sOBM polyclonal antibody ~~which recognizes~~recognizing mouse OBM ~~and an anti-human.~~ The

OBM/sOBM monoclonal antibody (H-OBM 9) which recognizes both human OBM and mouse OBM antigens were used as the antibodies in this example. Seven hundred microliters per well of each anti-OBM antibody were diluted serially stepwise with α -MEM containing 10% FCS and 350 μ l added to a 24 well plate (Nunc Co., Ltd.) in an amount of 700 μ l/well of, and male mouse splenocytes spleen cells suspended in the above medium (2×10^6 /ml) suspended in the same medium described above were also added to each well in a 24-an amount of 350 μ l/well plate (Nunc). Next Then, ST2 cells trypsinized and ST2 cells were suspended (8×10^4 cells/ml) in the above-mentioned culture medium containing 4×10^{-8} M Vitamin D₃ and 4×10^{-7} M Dexamethazone (8×10^4 cells/ml) were dexamethasone, and the resulting suspension was added to each well in the an amount of 350 μ l/well, followed by culturing for six. The plate was incubated at 37°C for 6 days at for 37°C culture. After the plates were washed once with PBS once, the cells in each well were fixed with a mixture of 50% ethanol and 50% acetone (50:50) for one hour at room temperature for a minute. The plates were After the plate was air-dried in air, and 500 μ l of substrate solution was added to each in an amount of 500 μ l/well according to their accordance with a protocol of the LEUKOCYTE ACID PHOSPHATASE a leukocyte acid phosphatase kit (Sigma Co., Ltd.), followed by incubating and the plate was left to stand at 37°C for 55 minutes at 37°C so as to cause reaction. Only the cells exhibiting the By this reaction, cell showing tartaric acid- resistant acid phosphatase activity (TRAP activity), which is a specific marker for of osteoclasts, were stained by this reaction. The plates were After the plate was washed once with distilled water once and air-dried in air, and the number of TRAP-positive cells was were counted. The results are shown in Table 4. As shown in Table 4, a result, it was found that both of the rabbit anti-mouse OBM/sOBM polyclonal antibody and the anti-human OBM/sOBM monoclonal antibody, H-OBM 9, which recognizes mouse OBM 9 inhibited derivation of OCL formation in a dose-dependent manner depending on the concentrations of the antibody. These It was found that these antibodies were found to possess had osteoclastogenesis- inhibitory activity like as in the case of an osteoclastogenesis-inhibitory factor, OCIF/OPG, and thus are promising were useful as a therapeutic agent medicament for treating bone metabolism abnormality symptoms.

TABLE 4

Table 4

Amount of antibody (μg/ml)	Number of TRAP-positive multinucleates	
	Rabbit anti-mouse OBM/sOBM polyclonal antibody	Mouse anti-human OBM/sOBM monoclonal antibody (H-OBM-9)
0	1155±53	1050±45
10	510±24	650±25
100	10±3	15±4

Amount of Antibody Added (ng/ml)	Number of TRAP-Positive Multinucleate Cells	
	Rabbit Anti-Mouse OBM/sOBM Polyclonal Antibody	Mouse Anti-Human OBM/sOBM monoclonal antibody (H-OBM-9)
0	1155 ± 53	1050 ± 45
10	510 ± 24	650 ± 25
100	10 ± 3	15 ± 4

(Average ± standard deviation, n = 3)

<[Example 39]>

Human osteoclast formation inducing activity of Trx-OBM

Osteoclastogenesis Inducing Activity of Trx-OBM

Mononuclear cells were prepared from whole blood collected from the vein of a healthy normal adult by density gradient human using Histopaque (Sigma Co., Ltd.) according to the protocol with density gradient technique in accordance with an attached thereto protocol. The mononuclear cells were suspended at a cell density concentration of 1.3×10^6 cells/ml in α -MEM containing 10^{-7} M Dexamethasone, 200 ng/ml of macrophage colony stimulating factor (The Green Cross Corp/Midori Juji Co., Ltd.), 10% fetal bovine serum, and stepwise concentration (0 to 100 ng/ml) of purified Trx-OBM (0-100 ng/ml) obtained in Example 15. The cell suspension was added to each well in a 48-well plates in the amount of 300 μ l per well, and the cells were cultured. The plate was incubated at 37°C for three days for culturing cells. After that, the culture broth medium was replaced with the new (identical with above-mentioned culture medium), and the cells were cultured. The plate was incubated at 37°C for four another 4 days. The cultured cells showing tartaric acid resistant acid phosphatase activity (TRAP activity) were selectively stained according to the method described in Example 5. The number of stained

multinucleated multinuclear cells was measured by counted under the microscope observation. The results are shown in Figure Fig. 29. It was confirmed that TRAP-positive multinucleated cells were induced in a dose-dependent manner by addition of Trx-OBM result, while no TRAP-positive cells showing TRAP activity were hardly detected in the wells to which containing no Trx-OBM, while TRAP positive multinuclear cells appeared in a manner depend on concentration of Trx-OBM when Trx-OBM was not added. Moreover Further, these TRAP-positive multinucleated cells showed positive result for vitronectin receptor which is a marker for osteoclasts. Furthermore In addition, when similar the cells same culture was carried out conditions were used on ivory dentin slices fragments placed on each well in a 48-well plate, pit formation was observed on the ivory slices absorption cavities were formed on the surface of dentin fragments only in the presence of Trx-OBM. Based on these findings, Trx-OBM was formed to have the Thereby, it was revealed that Trx-OBM had activity of inducing human osteoclast to induce formation of human osteoclasts.

<[Example 40]>

Inhibition of bone resorbing activity by anti-OBM/sOBM antibody

Bone Resorption Inhibitory Activity of Anti-OBM/sOBM Antibody

15-day pregnant ddY mice (Nippon SLC Co., Ltd.), 25 μ Ci of [45 Ca] -CaCl₂ solution (Amersham Co.) was, Ltd.) were injected subcutaneously injected to ddY mouse (Japan SLC Co.) in the 15th day of pregnancy at a dose of 25 μ Ci per mouse to label the bone of the fetus, and fetal bones were labeled with 45 Ca. Next On the following day, the mouse was sacrificed mice were slaughtered, and their abdomens were opened to obtain remove fetuses from the fetus uterus. The forefoot of the fetus was drawn and A forelimb was removed from the fetus, the skin and muscle were removed to obtain the take out a long bones. The bone, and a cartilage on the long bone was also removed so as to obtain leave only the shafts diaphysis of the long bones. The shafts of long bones were Each diaphysis was floated one by one in 0.5 ml of culture medium (BGJb medium (GIBCO-BRL company Gibco Co., Ltd.) containing a 0.2% bovine serum albumin (Sigma Co., Ltd.)) in each well in 24-well plates, and cultured for 24 hours at 37°C in the presence of 5% CO₂ for 24 hours. After completion of the pre-cultivation culture, the bones were long bone was transferred into various fresh to a new culture medium (0.5

ml), each containing one of different various bone resorbing factors (vitamin D₃, prostaglandins E₂, parathyroid hormone, interleukin 1- α), and normal rabbit IgG (100 μ g/ml; as a control), or the rabbit anti-OBM/sOBM polyclonal antibody prepared obtained in Example 28, followed by further cultivation and then cultured for another 72 hours. After completion of the cultivation culture, the long bones were placed in was put into 0.5 ml of an aqueous solution of 5% trichloroacetic acid aqueous solution (Wako Pure Chemicals Co. Industries, Ltd.), and allowed to stand treated at room temperature for more at than least 3 hours so as to decaify be decalcified. Five To the conditioned medium and the trichloroacetic acid extract (0.5 ml each), 5 ml of a scintillator (AQUASOL-2, PACKARD Packard Co., Ltd.) was added to the culture broth and the extract of the trichloroacetic acid solution (each 0.5 ml) to measure the radioactivity of ⁴⁵Ca, whereby the ratio was measured. The proportion of the ⁴⁵Ca which was liberated inte in the culture broth by solution due to bone resorption was calculated. The results are shown in Figures Figs. 30 to 33. As a result, although the vitamin D₃ (10⁻⁸ M) was found to caused increase the of bone resorbing activity, but the bone resorption caused by the vitamin D₃ was inhibited by addition of the rabbit anti-OBM/sOBM polyclonal antibody suppressed the bone resorption stimulated by vitamin D₃ in a concentration-dependent manner, and the bone resorption was completely inhibiting inhibited by addition of the increased bone resorption antibody at a concentration of 100 μ g/ml (Figure 30). Prostaglandins Fig. 30). Further, although bone resorption activity was increased in the presence of prostaglandin E₂ (10⁻⁶ M) and or the parathyroid hormone (100 ng/ml) also increased the bone resorbing activity. However, the bone resorption caused by the prostaglandin E₂ or the parathyroid hormone was almost completely inhibited by the addition of 100 μ g/ml of the rabbit anti-OBM/sOBM polyclonal antibody almost completely inhibited the bone resorption stimulated by prostaglandins E₂ and parathyroid hormone (Figures 100 μ g/ml)(Figs. 31 and 32). On Meanwhile, the other hand, normal rabbit IgG (100 μ g/ml), which was used as a positive control, did not affect the bone resorbing activity induced by prostaglandins E₂ and had no effects on the bone resorption by the prostaglandin E₂ and the parathyroid hormone. Bone Further, although bone resorption was also increased by induced by the interleukin 1- α (10 ng/ml), but as well, the bone resorption was inhibited significantly

inhibited by the addition of rabbit anti-OBM/sOBM polyclonal antibody (100 µg/ml) (Figure 23). Based on these results, it is clear that the antibody of the present invention is excellent as a superior bone resorption inhibitory substance as a bone resorption inhibitor. The results obtained by similar experiments using H-OBM 9 which is a mouse anti-human OBM/sOBM antibody, it was confirmed that this antibody exhibits an almost H-OBM 9 had approximately equivalent bone resorption-inhibitory effect as activity to that of the rabbit anti-OBM/sOBM polyclonal antibody.

Industrial Applicability

The present invention provides a novel protein that specifically binds to osteoclastogenesis-inhibitory factor (OCIF), a process method for preparing the protein production thereof, a method for screening method for a substance which controls expression of this protein using this by use of the protein, a screening method for screening a substance which inhibits or modulates the activity of this protein, a screening method for screening a receptor which binds the protein and transmits the activity of this protein by binding thereto thereof, a pharmaceutical composition which contains the comprising a substance obtained by these said method for screening methods, an antibody for the said protein, and an agent for treating bone metabolism abnormality which is formulated using the antibody.

Moreover, the present invention provides a DNA encoding. Furthermore, the present invention provides a DNA which encodes a novel protein (OCIF-binding molecule) which binds to osteoclastogenesis-inhibitory factor (OCIF), a protein which possesses having an amino acid sequence encoded by the DNA, a method for preparing the method for genetically producing a protein which specifically binds to the OCIF using said by use of the DNA by a genetic engineering technique, and an agent for treating bone metabolism comprising said the protein for treating bone metabolism acatastasia. Furthermore, the present invention provides a Moreover, methods are provided for screening method for a substance which controls the expression of the OCIF-binding molecule, a screening method for screening a substance which binds to the OCIF binding molecule and inhibits or modulates the activity of the OCIF-

~~binding molecule by binding thereto thereof, a screening method for the screening a~~
~~receptor which binds OCIF binding molecule and transmits the activity of the OCIF-~~
~~binding molecule by binding thereto thereof, and a pharmaceutical composition which~~
~~contains the comprising a substance obtained by the said method for screening methods.~~

Still further, the present invention provides a DNA encoding Also provided is:
DNA, which encodes a novel human protein capable of binding to osteoclastogenesis-
inhibitory factor, OCIF (human-derived OCIF- binding molecule, human OBM) which
binds osteoclastogenesis inhibitory factor (OCIF), a protein containing and having an
amino acid sequence encoded by the DNA, a process method for preparing genetically
producing a protein having characteristics of which specifically binding to binds OCIF and
exhibiting has a biological activity to support and promote the osteoclast differentiation
and maturation of osteoclasts by means use of genetic engineering technique the DNA, and
an agent for treating bone metabolism abnormality using comprising the protein.

Furthermore, the present invention provides

Also provided are: a screening method for screening a substance which controls
expression of the OCIF- binding molecule, a screening method for screening a substance
which binds the OCIF binding molecule and inhibits or modulates the activity of the
OCIF binding molecule by binding thereto thereof, a screening method for the screening a
receptor which binds the OCIF binding molecule and transmits the biological activity of
the OCIF binding molecule by binding thereto thereof, and a pharmaceutical composition
which contains the comprising a substance obtained by the said method for screening
methods, as well as an antibody to the human-derived OCIF- binding protein, and an
agent for preventing and/or treating bone metabolism abnormality symptoms which is
formulated using the antibody.

In addition Moreover, the present invention provides antibodies an antibody (anti-
OBM/sOBM antibody) which recognizes both of the following antigens (anti-
OBM/sOBM antibodies), one is i.e., a membrane-bound protein binding molecule (OCIF
binding molecule; OBM) which specifically binds to an OCIF (OCIF binding molecule;
OBM), and the other a soluble OBM (sOBM) which does not have a lacking membrane
binding regionsites, a process method for preparing production of the antigen body, a
method for measuring the OBM and sOBM using these antibodies by use of the antibody,

and an agent for preventing and/or treating bone metabolism abnormality symptoms which using comprise the antibody as an effective active component ingredient.

The proteins and or antibodyies prepared by the process of the present invention are useful as medicines and/or medicaments, experimental reagents or diagnostic reagents for research and test purposes.

Description of deposited microorganisms

(1) ~~Name and address of the depository organization to which microorganism was deposited~~

Reference to Deposited Microorganisms

(1) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry
1-3,1-3 Higashi-1-Chome, Tsukuba-shi, Ibaraki-ken, Japan (postal code (zip: 305)

~~Date of deposition to the depository organization~~ Deposit:

May 23, 1997

~~The deposition number~~

Deposit Number:

FERM BP-5953

(2) ~~Name and address of the depository organization to which microorganism was deposited~~

(2) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry
1-3,1-3 Higashi-1-Chome, Tsukuba-shi, Ibaraki-ken, Japan (postal code zip: 305)

~~Date of deposition to the depository organization~~ Deposit:

August 13, 1997

~~The deposition number~~

Deposit Number:

FERM BP-6058

(3) — ~~Name and address of the depository organization to which microorganism was deposited~~

(3) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry
1-3,1-3 Higashi-1-Chome, Tsukuba-shi, Ibaraki-ken, Japan(postal code(zip: 305)

~~Date of deposition to the depository organization~~Deposit:

November 5, 1997(~~Original deposition date~~)

~~The deposition number~~

Deposit Number:

FERM BP-6264

(4) — ~~Name and address of the depository organization to which microorganism was deposited~~

(4) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry
1-3,1-3 Higashi-1-Chome, Tsukuba-shi, Ibaraki-ken, Japan(postal code(zip: 305)

~~Date of deposition to the depository organization~~Deposit:

November 5, 1997(~~Original deposition date~~)

~~The deposition number~~

Deposit Number:

FEPRM BP-6265

(5) — ~~Name and address of the depository organization to which microorganism was deposited~~

(5) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry

1-3,1-3 Higashi-1-Chome, Tsukuba-shi, Ibaraki-ken, Japan(postal code(zip: 305)

Date of ~~deposition to the depository organization~~Deposit:

November 5, 1997(~~Original deposition date~~)

~~The deposition number~~

Deposit Number:

FERM BP-6266

5

NOVEL PROTEIN AND METHOD FOR PRODUCTION THEREOF

5 TECHNICAL FIELD

 The present invention relates to a novel protein (OCIF binding molecule; hereinafter it may be referred to as "OBM") which binds osteoclastogenesis inhibitory factor, and a production method thereof.

10 Furthermore, the present invention also relates to DNA which encodes the protein, a protein having an amino acid sequence encoded by the DNA, a method for genetically producing the protein, and a pharmaceutical composition comprising the protein.

15 Moreover, the present invention also relates to a methods for screening a substance which controls expression of the protein, a substance which inhibits or modifies the biological activity of the protein, or, a receptor which binds the protein and transmits activity thereof, using the protein
20 or the DNA, the substances obtained by these methods, and pharmaceutical compositions comprising the obtained substances.

 Furthermore, the present invention also relates to an antibody to the protein, a method for the production thereof,
25 a method for measuring the protein with the antibody, and an agent comprising the antibody.

BACKGROUND ART

 Bone metabolism depends on the overall activity of
30 osteoblasts responsible for bone formation and osteoclasts responsible for bone resorption. It is assumed that bone metabolism abnormality is caused due to loss of balance between bone formation and bone resorption. As diseases involving in bone metabolism abnormality, osteoporosis,
35 hypercalcemia, bone Paget's disease, renal osteodystrophy, rheumatoid arthritis and osteoarthritis are known. A representative of these bone metabolism abnormality diseases is osteoporosis. This disease occurs when bone resorption by osteoclasts exceeds bone formation by osteoblasts and is

characterized by equal decrease in bone calcareous substances and bone matrix. The mechanism for crisis of this disease is not yet fully clarified, while it is a disease with pain in bone and bone fracture due to the increased fragility of bone.

5 Along with an increase in population of aged people, this disease causes aged people to bone fracture, resulting in letting them fixed on bed. This disease has already been a social problem, so that medicaments for treating the disease is urgently needed to be developed. It is expected that
10 osteopenia due to bone metabolism abnormality can be treated by stimulating bone formation, inhibiting bone resorption, or, improving the balance between them. That is, bone formation is expected be stimulated by promoting the growth, differentiation and functions of osteoblast which is
15 responsible for bone formation, suppressing the differentiation of osteoclast precursor cells to osteoclasts and maturation thereof, or suppressing osteoclast function such as bone-resorbing activity. At present, hormones, substances of low molecular weight or physiologically active
20 proteins having such activity are remarkably studied and developed.

As agents for treating bone-relating diseases and shortening treating periods thereof, a calcitonin-containing formulation, active-form vitamin D₃-containing formulation,
25 hormone (estradiol, ipriflavone, vitamin K₂) -containing formulation and bisphosphonate-based compound have already been clinically available. Furthermore, to develop medicaments with less side effects and excellent effectiveness, clinical trials of active-form vitamin D₃
30 derivatives, estradiol derivatives, and bisphosphonate-based compounds of the second or third generation have been held.

However, since such methods for treating using these drugs are not necessarily sufficient in effectiveness and results of treatment, novel medicaments that are safer and
35 have higher effectiveness has been expected to be developed. Moreover, among medicaments used in treatment of bone metabolism diseases, there are those which can used only for treating a restricted kind of diseases due to side effects thereof. In addition, at present, to treat bone metabolism

diseases such as osteoporosis, treatment with combined use of more than one medicament is concurrently usual. From such a point of view, a medicament having different action mechanisms from those of the conventional one with higher effectiveness and less side effect has been expected to be developed.

As described above, cells responsible for bone metabolism are osteoblasts and osteoclasts. It is known that these cells closely interact with each other, and this phenomenon is regarded as coupling. That is, it has been reported that the differentiation and maturation of osteoclasts are stimulated or suppressed by cytokines, interleukins 1 (IL-1), 3 (IL-3), 6 (IL-6) and 11 (IL-11), granulocyte-macrophage colony-stimulating factors (GM-CSF), macrophage colony-stimulating factors (GM-CSF), interferon gammas (IFN- γ), tumor necrosis factors α (TNF- α), transforming growth factors β (TGF- β) and the like, which are secreted from osteoblast-like stroma cells (Raisz: Disorders of Bone and Mineral Metabolism, 287 to 311, 1992; Suda et al.: Principles of Bone Biology, 87 to 102, 1996; Suda et al.: Endocrine Reviews, 4, 266 to 270, 1995, Lacey et al.: Endocrinology, 136, 2369 to 2376, 1995). It is known that the osteoblast-like stroma cells play an important role in differentiation and maturation of osteoclasts and expression of mature osteoclast's function such as bone resorption through intercellular binding to immature precursor cells of osteoclast or (mature) osteoclasts. As a factor involved in osteoclastogenesis by the intercellular binding, a molecule known as osteoclast differentiation factor (ODF) (Suda et al.: Endocrine Rev. 13, 66 to 80, 1992; Suda et al.: Bone 17, 87S to 91S, 1995) which is expressed on the membrane of the osteoblast-like stroma cell is predicted. According to this assumption, a receptor for ODF exists in the osteoclast precursor cell. However, ODF and this receptor are not yet either purified or identified, and there are no reports on their characteristics, action mechanisms and structures. As just described, the mechanism for differentiation and maturation of osteoclast has not been fully understood yet, and it is expected that full understanding of that mechanism will significantly contribute not only to the field

of experimental medicines but also to developments of novel agents for treating bone metabolism abnormality, based on the novel action mechanism.

Under the circumstances, the present inventors have made intensive studies and found osteoclastogenesis inhibitory factors (OCIF) in the culture solution of human fetal lung fibroblasts IMR-90 (ATCC CCL186) (W096/26217).

Then, the present inventors have succeeded in DNA cloning of OCIF, production of a recombinant OCIF using an animal cell, and confirmation of in vivo medicinal virtues (bone metabolism improving effect) of the recombinant OCIF. OCIF is expected as a medicament that has higher effectiveness and causes less side effects than the conventional one and can prevent and treat diseases associated with bone metabolism abnormality.

DISCLOSURE OF THE INVENTION

The present inventors have intensively searched the existence of a protein binding to osteoclastogenesis inhibitory factor OCIF by using OCIF. As a result, the inventors have found that OCIF binding protein is specifically expressed on an osteoblast-like stroma cell cultured in the presence of bone resorption factors such as active-form vitamin D₃ and parathyroid hormone (PTH). Furthermore, as a result of studying the characteristics and physiological functions of OCIF binding protein, the protein has found to have biological activity as a so-called osteoclast differentiation and maturation factor, associated with differentiation of immature osteoclast precursor cells to osteoclast and maturation thereof. The present invention has been completed based on this finding. Moreover, as a result of further studying the protein of the present invention, the present inventors have found that the novel membrane protein is an important protein which leads the differentiation and maturation of immature osteoclast precursor cells to osteoclasts by osteoblast-like stroma cells in a co-culture system of the osteoblast-like stroma cells and spleen cells. The successful identification, isolation and purification of the protein as a factor which supports or promotes the

differentiation and maturation of osteoclast in the present invention enables a screening of a novel agent for treating bone metabolism abnormality, based on mechanism for bone metabolism in a living subject, using the protein of the present invention.

Therefore, an object of the present invention is to provide a novel protein (OCIF binding molecule; OBM) which binds osteoclastogenesis inhibitory factor OCIF, and a method for the production thereof. Another object of the present invention is to provide DNA which encodes the protein, a protein having an amino acid sequence encoded by the DNA, a method for genetically producing the protein, and a pharmaceutical composition comprising the protein. Furthermore, another object of the present invention is to provide an agent for preventing and/or treating bone metabolism abnormality comprising the protein. Moreover, another object of the present invention is to provide a method for screening a substance which controls expression of the protein, a substance which inhibits or modifies the biological activity of the protein, or, a receptor which binds the protein and transmits the activity of the protein, using the protein and DNA thereof, a substance obtained by that method, and, pharmaceutical compositions comprising the obtained substance. Furthermore, another object of the present invention is to provide an antibody to the protein, a method for production thereof, a method for measuring the protein using the antibody, and a medicament (agent; pharmaceutical composition) comprising the antibody.

The protein of the present invention shows the following physicochemical properties and biological activity. That is, (a) the protein specifically binds osteoclastogenesis inhibitory factor (OCIF) and has high affinity (a dissociation constant, a K_d value, on a cell surface, is not larger than 10^{-9} M); (b) the protein shows a molecular weight of about 30,000 to 40,000 as measured by SDS-polyacrylamide electrophoresis under non-reducing conditions, and shows an apparent molecular weight of about 90,000 to 110,000 when crosslinked with a monomer-type OCIF; and

(c) the protein has an activity to support or promote the differentiation and maturation of osteoclast in co-culture of mouse osteoblast-like stroma cell and mouse spleen cell in the presence of bone resorption factors such as active-form vitamin D₃ and parathyroid hormones (PTH).

As a representative in vitro culture system for osteoclastogenesis, a co-culture system of mouse-derived osteoblast-like stroma cell line, ST2, and mouse spleen cells in the presence of an active-form vitamin D₃ or PTH is well known. The cells that express the protein of the present invention can be obtained by examining the binding ability of a mouse osteoblast-like stroma cell or mouse spleen cell cultured in the presence or absence of active-form vitamin D₃ to OCIF. The protein of the present invention is identified as a protein which is specifically induced on an osteoblast-like stroma cell cultured in the presence of bone resorption factors such as active-form vitamin D₃ or PTH. Further, in consideration of the following facts that osteoclast formation is inhibited by addition of OCIFs to the above co-culture system in the presence of the active-form vitamin D₃, within a range of 1 to 40 ng/ml of OCIF in a dose-dependent manner, that there is an intimate correlation between change in expression of the present protein induced on the ST2 cells in the presence of active-form vitamin D₃ and change in osteoclast formation with the passage of time, that the amount of the present protein expressed on ST2 cell corresponds the intensity of an ability of support osteoclast formation, and that osteoclast formation is completely inhibited by binding of OCIFs to the present protein on the ST2 cells, the protein of the present invention is identified as a protein having biological activity (effect) to support or promote the differentiation and maturation of osteoclast.

The affinity of the protein of the present invention for OCIF can be assessed by labeling OCIF and testing the binding activity of the labeled OCIF to the surface of an animal cell membrane. OCIF can be labeled by a commonly used protein-labeling method such as labeling with a radioisotope or fluorescence labeling. For instance, an example of labeling OCIF with a radioisotope is ¹²⁵I labeling at a tyrosine

residue, and labeling methods such as Iodogen method, chloramine T method and enzyme method can be employed thereto. The binding activity of the thus labeled OCIF to the surface of an animal cell membrane can be examined in accordance with a commonly used method, an amount of nonspecific binding can be measured by adding 100 to 400 times excess amount of unlabeled OCIF to the medium for binding experiment. The amount of specific binding of OCIF is calculated by subtracting that of the nonspecific binding from that of total binding. The affinity (for OCIF) of the present protein expressed on a cell membrane is assessed by conducting the test with various amounts of the labeled OCIF and analyzing the amount of the specific binding by Scatchard plot. The determined affinity of the protein of the present invention for OCIF is about 100 to 500 pM. Thus, the protein of the present invention is identified as a protein having such high affinity (a dissociation constant, a Kd value, on a cell membrane is not larger than 10^{-9} M) for OCIF. The molecular weight of OBM is measured by use of gel filtration chromatography, SDS-PAGE or the like. To measure the molecular weight more accurately, SDS-PAGE is preferably used, and OBM is identified as a protein having a molecular weight of about 40,000 ($40,000 \pm 4,000$) under reducing conditions.

The protein of the present invention can be obtained from a mouse osteoblast-like stroma cell line, ST2, a mouse fat cell strain PA6, or human osteoblast-like cell lines, or concentrated osteoblast-like cells obtained from mammals such as human, mouse and rat. And, substances that are required to express the protein of the present invention on these cells may be bone resorption factors such as active-form vitamin D₃ (Calcitriol), parathyroid hormone (PTH), interleukins (IL)-1, IL-6, IL-11, oncostatin M, and leukemia cell growth inhibiting factor (LIF). As for the amounts of these substances, it is desirable to use the active-form vitamin D₃ or PTH in an amount of 10^{-8} M; the IL-11 and the oncostatin M in amounts of 10 ng/ml and 1 ng/ml, respectively; and the IL-6 in an amount of 20 ng/ml with 500 ng/ml of IL-6 soluble receptor. It is preferable to use cells obtained by culturing mouse osteoblast-like stroma cell line, ST2, in α -MEM containing 10^{-8}

⁸ M active-form vitamin D₃, 10⁻⁷ M dexamethasone and 10% bovine fetal serum for at least one week until the cells become confluent. Thus cultured cells can be removed and collected by using a cell scraper or the like. Moreover, the collected
5 cells can be stored at -80°C until use.

The protein of the present invention can be purified efficiently from a membrane fraction of the thus collected cells. The membrane fraction can be prepared in accordance with a common method used for fractionation of organelle. As
10 a buffer used in preparation of the membrane fraction, various protease inhibitors may be preferably added. Illustrative examples of protease inhibitors to be added include serine protease inhibitors, thiol protease inhibitors, and metalloprotease inhibitors, such as PMSF, APMSF, EDTA, O-phenanthroline, leupeptin, pepstatin A, aprotinin and a
15 soybean trypsin inhibitor. To crush the cells, Daunce homogenizer, polythoron homogenizer, an ultrasonicator or the like can be used. The crushed cells can be suspended in a buffer containing 0.5 M sucrose and centrifuged at 600 X g for
20 10 minutes so as to separate cell nuclei and uncrushed cells as a precipitated fraction. After further centrifuging at 150,000 X g for 90 minutes, a membrane fraction can be obtained as a precipitated fraction. By treating the thus obtained membrane fraction with various surfactants, the
25 protein of the present invention existing on the cell membrane can be solubilized and extracted, efficiently. For solubilization, various surfactants which are conventionally used in solubilization of cell membrane proteins, such as CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), Triton X-100, Nikkol and n-octylglycoside,
30 can be used. The protein of the present invention is preferably solubilized by adding 0.5% CHAPS to the protein and agitating the mixture at 4°C for 2 hours. By centrifuging the thus prepared sample at 150,000 X g for 60 minutes, the
35 supernatant can be obtained as a solubilized membrane fraction.

The protein of the present invention can be purified efficiently from the thus obtained solubilized membrane fraction, using an OCIF-immobilized column, gel or resin. As

OCIF used in the immobilization, that isolated from the culture solution of human fetal lung fibroblasts IMR-90 in accordance with a method described in WO96/26217 or that obtained by gene engineering (rOCIF) can be used. This rOCIF can be obtained by incorporating the corresponding human, rat or mouse cDNA into an expression vector in accordance with a common method, expressing the rOCIF with animal or insect cells such as CHO cells, BHK cells and Namalwa cells, and then purifying it. The thus obtained OCIF shows a molecular weight of about 60 kDa (monomer type) and a molecular weight of about 120 kDa (dimer type). A dimer type OCIF is preferably used in the immobilization. As gel or a resin for immobilizing OCIF, ECH sepharose 4B, EAH sepharose 4B, thiopropyl sepharose 6B, CNBr-activated sepharose 4B, activated CH sepharose 4B, epoxy activated sepharose 6B, activated thiol sepharose 4B (products of Pharmacia Co., Ltd.), TSKgel AF-epoxy TOYOPAL 650, TSKgel AF-amino TOYOPAL 650, TSKgel AF-formyl TOYOPAL 650, TSKgel AF-carboxy TOYOPAL 650, TSKgel AF-Tresyl TOYOPAL 650 (products of TOSO CO., LTD.), amino-CELLULOFINE, carboxy-CELLULOFINE, FMP activated CELLULOFINE, formyl-CELLULOFINE (products of SEI KAGAKU KOUGYO CO., LTD.), AFFIGEL 10, AFFIGEL 15 and AFFIPREP 10 (products of BioRad Co., Ltd.) are available. Furthermore, as a column for immobilizing OCIF, a HiTrap NHS-activated column (Pharmacia Co., Ltd.), TSKgel Tresyl-5PW (TOSO CO., LTD.) or the like can be used. As a specific example of a method for immobilizing OCIF with the HiTrap NHS-activated column (1 ml, Pharmacia Co., Ltd.), the following method can be presented. That is, 1 ml of 0.2 M NaHCO_3 /0.5 M NaCl (pH: 8.3) solution containing 13.0 mg of OCIF is applied to the column and allowed to undergo a coupling reaction at room temperature for 30 minutes. Then, after 0.5 M ethanolamine/0.5 M NaCl (pH: 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH: 4.0) are applied, respectively, 0.5 M ethanolamine/0.5 M NaCl (pH: 8.3) is applied again, and then the column is left to stand at room temperature for 1 hour so as to inactivate an excess amount of active groups. Thereafter, the column is washed twice with 0.5 M ethanolamine/0.5 M NaCl (pH: 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH: 4.0), and then replaced with 50 mM Tris/1 M

NaCl/0.1% CHAPS buffer (pH: 7.5). Finally, an OCIF-immobilized column can be prepared. Using the prepared OCIF-immobilized column, gel or resin, the protein of the present invention can be purified efficiently. To prevent the proteolysis of the protein of the present invention, the above various protease inhibitors may also be added to the buffer solution used in purification. After applying the above solubilized membrane fraction to an OCIF-immobilized column, or, mixing the solubilized membrane fraction with an OCIF-immobilized gel or resin and subsequently stirring the mixture so as to cause the fraction to be adsorbed, the protein of the present invention can be eluted from the OCIF-immobilized column, gel or resin, using an acid, various protein-denaturing agents, a cacodylate buffer or the like. To minimize denaturation of the protein of the present invention, it is preferred to neutralize eluate immediately using an acid. As an acid buffer solution used for elution, 0.1 M glycine-hydrochloric acid buffer solution (pH: 3.0), 0.1 M glycine-hydrochloric acid buffer solution (pH: 2.0) and 0.1 M sodium citrate buffer solution (pH: 2.0) can be used, for example.

Thus purified protein of the present invention can be further purified by use of a method which is conventionally employed in purification of proteins from biological samples, through various purification operations taking advantages of the physicochemical properties of the protein of the present invention. To concentrate a solution of the protein of the present invention, a method which is conventionally used in protein purification process, e.g., ultrafiltration, freeze-drying and salting-out, can be used. Preferably, ultrafiltration based on centrifugation with Centricon-10 (BioRad Co., Ltd.) and the like is preferably used. Furthermore, as purification means, various methods conventionally used in protein purification using ion exchange chromatography, gel filtration chromatography, hydrophobic chromatography, reversed phase chromatography, preparative electrophoresis and the like can be used in combination. More specifically, the protein of the present invention can be purified by concombined use of gel filtration chromatography with Superose 12 column (Pharmacia Co., Ltd.) and the like,

and, reversed phase chromatography. Moreover, the protein of the present invention during the purification process can be detected by analyzing activity to bind the immobilized OCIF, or by immuno-precipitation of OCIF-binding substances with an anti-OCIF antibody followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

The thus obtained protein of the present invention is useful, due to its activity, as medicaments, e.g., as agents for treating bone metabolism abnormality such as osteopetrosis, or experimental and diagnostic reagents.

Furthermore, the present invention relates to DNA which encodes a novel protein (OCIF binding molecule; OBM) which binds osteoclastogenesis inhibitory factor (OCIF), a protein having an amino acid sequence encoded by the DNA, a method for genetically producing a protein which specifically binds OCIF by use of the protein, and an agent for treating bone metabolism abnormality comprising the protein. In addition, the present invention relates to a method for screening a substance which controls expression of OBM, a method for screening a substance which binds OBM and inhibits or modifies an effect thereof, a method for screening a receptor which binds OBM and transmits an effect thereof, and pharmaceutical compositions comprising a substance obtained as a result of these methods for screening.

The novel protein OBM encoded by the DNA of the present invention shows the following physicochemical properties and biological activity. That is,

(a) the protein specifically binds osteoclastogenesis inhibitory factor (OCIF),

(b) the protein shows a molecular weight of about 40,000 (\pm 4,000) as measured by SDS-PAGE under reducing-conditions, and shows an apparent molecular weight of about 90,000 to 110,000 when crosslinked with a monomer-type OCIF, and

(c) the protein has an activity to support or promote differentiation and maturation of osteoclast.

Human osteoclastogenesis inhibitory factor (OCIF) is used as a probe for assessing the properties of OBM in identification of the DNA encoding OCIF binding molecule OBM of the present invention, and can be isolated from the culture

solution of human fetal lung fibroblasts IMR-90 in accordance with WO96/26217. For isolation and identification of the DNA encoding OBM, recombinant human OCIF, recombinant mouse OCIF, recombinant rat OCIF and the like can also be used. This
5 recombinant OCIF can be obtained by incorporating the corresponding DNA into an expression vector in accordance with a commonly used method, subsequently expressing OCIF with animal or insect cells such as CHO cells, BHK cells and Namalwa cells, and then purifying it.

10 As a method for cloning a cDNA which encodes the target protein (cDNA cloning), a method comprising the steps of determining a partial amino acid sequence of the protein and isolating the target cDNA by hybridization method based on a Nucleotide Sequence corresponding to the amino acid
15 sequence, and another method which comprises the steps of constructing a cDNA library with an expression vector, regardless of whether or not the amino acid sequence of the protein is known, subsequently introducing it into cells, and then screening the presence and absence of expression of the
20 target protein and isolating the desired cDNA (D' Andrea et al.: Cell 57, 277 to 285, 1989; Fukunaga et al.: Cell 61, 341 to 350, 1990) (expression cloning method) is also available. In the expression cloning method, bacterial, yeast and animal cells and the like are selected and used as host cells
25 according to purposes. For cloning a cDNA which encodes a protein considered to present on the surface of animal cell membrane as in the present invention, animal cells are often used as hosts. Furthermore, hosts with high efficiency for introducing DNA and expressing the introduced DNA are
30 conventionally used. One of cells having such characteristics is a monkey kidney cell line, COS-7 cell, used in the present invention. Since SV40 large T antigen is expressed in COS-7 cell, plasmids having SV40 replication origin are present in the cell as multicopy episome, whereby higher expression than
35 usual can be expected. Moreover, since the maximum expression level is reached within a few days after the introduction of DNA, COS-7 cells are suitable for quick screening. In combination with a plasmid suitable for high expression, this host cell enables an extremely high level of gene expression.

Promoter is a factor of a plasmid which has the most significant effects on the amount of gene expression. As a promoter suitable for high level of expression, SR α promoter and cytomegalovirus-derived promoter are often used. As a
5 screening method for cloning the cDNA of the membrane protein by expression cloning, binding method, panning method and film emulsion method are available.

The present invention relates to DNA, which encodes the protein which specifically binds OCIF (OBM), is obtained
10 by a combination of the expression cloning method and the binding method, the protein expressed therewith, and a screening of a biologically active substance with the DNA or the protein. OBM encoded by the DNA of the present invention can be detected by labeling OCIF and subsequently examining
15 the binding activity of the labeled OCIF to the surface of an animal cell membrane. OCIF can be labeled by a conventional method for labeling protein such as labeling with a radioisotope or fluorescence labeling. An example of labeling OCIF with radioisotope is ¹²⁵I labeling at tyrosine residue, and
20 specific labeling methods include Iodogen method, chloramine T method and enzyme method. The binding activity of thus labeled OCIF to the surface of an animal cell membrane can be assessed in accordance with a commonly used method. Furthermore, an amount of nonspecific binding can be measured
25 by adding an 100 to 400 times excess amount of unlabeled OCIF to a medium for binding experiment. The amount of specific binding of OCIF is calculated by subtracting that of the nonspecific binding from that of total binding.

Based on an assumption that a factor, which is
30 involved in differentiation of osteoclast, interacts with OCIF, the inventors have screened an expression library prepared from the mRNA of a mouse osteoblast-like stroma cell line, ST2, with recombinant OCIF in accordance with the following method in order to separate the protein which binds
35 OCIF. DNA synthesized from the mRNA of the ST2 was inserted into an expression vector for an animal cell, and they were transduced (transfected) to a monkey kidney cell, COS-7. Using a ¹²⁵I-labeled OCIF as a probe, the target protein expressed on the COS-7 cell was screened. As a result, DNA

which encodes the protein that specifically binds OCIF could be separated, and then the Nucleotide Sequence of the DNA which encodes this OCIF binding molecule (OCIF binding molecule; OBM) was determined. Furthermore, it has been found that OBM encoded by the DNA strongly and specifically binds OCIF on the cell membrane.

An example of DNA hybridization under relatively mild conditions in the present invention is that after DNA is transferred to a nylon membrane and fixed in accordance with a common method, it is hybridized with a radio-labeled DNA as a probe in a hybridization buffer at 40 to 70°C for about 2 hours to 1 night, and then washed with 0.5 X SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 45°C for 10 minutes. More specifically, after DNA is transferred and fixed to a nylon membrane, HIBOND N (Amersham Co., Ltd.), in accordance with a conventional method, it is hybridized with a ³²P-labeled DNA as a probe in Rapid Hybridization Buffer (Amersham Co., Ltd.) at 65°C for 2 hours, and then washed with 0.5 X SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 45°C for 10 minutes.

As a representative in vitro culture system for osteoclastogenesis, a co-culture system of mouse-derived osteoblast-like stroma cell line, ST2, and mouse spleen cells in the presence of active-form vitamin D₃ or PTH is well known. OBM of the present invention is identified as a protein which is specifically induced on an osteoblast-like stroma cell cultured in the presence of bone resorption factors such as active-form vitamin D₃ and PTH. Furthermore, since osteoclasts formation is stimulated by adding the protein encoded by the DNA of the present invention to a culture system of mouse spleen cells even in the absence of active-form vitamin D₃ or PTH, OBM encoded by the DNA of the present invention is considered to be involved in differentiation and maturation of osteoclast.

A recombinant OBM can be produced by inserting the DNA of the present invention into an expression vector so as to prepare a plasmid for expressing OBM, and then introducing and expressing the plasmid in various cells and microbial strains. COS-7, CHO, Namalwa and the like can be used as mammalian

hosts cells for expression, and Escherichia coli (E. coli) and the like can be used as bacterial host cells for expression. In such a case, the recombinant OBM can be expressed as a membrane-bound protein using full length of DNA, or, as a secretory-type or solubilized-type (soluble-type) protein by removing a part of the DNA encoding a membrane-binding domain from the full length. Thus produced recombinant OBM can be purified efficiently in combination of conventionally methods used in protein purification such as affinity chromatography using an OCIF-immobilized column, ion exchange chromatography, gel filtration chromatography and the like. The thus obtained protein of the present invention is useful, due to its activity, as medicaments, e.g., as agents for treating bone metabolism abnormality such as osteopetrosis, and as experimental or diagnostic reagents.

The protein OBM encoded by the DNA of the present invention enables (1) screening of a substance which controls expression of OBM; (2) screening of a substance which specifically binds OBM and inhibits or modifies the biological activity of OBM; and (3) screening of a protein (OBM receptor) which exists on a precursor cell of osteoclast and transmits the biological activity of OBM, as well as developments of antagonists and agonists using this OBM receptor. In combinatorial chemistry using the above OBM or OBM receptor, a peptide library required to identify an antagonist or agonist can be prepared in accordance with the following specific methods. One of them is split method (Lam et al.; Nature 354, 82 to 84, 1991). In this method, synthetic carriers (beads) are bound to amino acids (units), separately. Then, these synthetic beads are mixed together and divided into an equal number of the units, and then bound to the subsequent units. By repeating this operation n times, a library in which n of units are bound to the carries is prepared. Such an operation allows the synthesis of only one sequence per one group of the carriers. Hence, when a positive carrier group is selected in said method for screening by use of the protein of the present invention and then the amino acid sequence thereof is determined, a peptide specifically binding can be identified. Further, as another method, phage display method can be used.

In this method, synthetic genes encoding random peptides are expressed with phage. While this method has an advantage that it can archive a larger number of molecules in a library than the above synthetic library, it also has a disadvantage that the kind of peptides per molecule is not so varied as the peptides having the sequences that phage doesn't prefer do not exist in the library. In phage display method, as in the case of split method, using a screening system with the protein of the present invention, phage specifically binding thereto is concentrated by panning, and thus obtained phage is amplified in *E. coli*, and, further, the Nucleotide Sequence encoding the peptide is determined. Furthermore, when it is desired that a specific peptide having high affinity for OBM or OBM receptor is screened from a peptide library using the screening system of the above (2) or (3), specific peptide having very high affinity can be obtained by screening a positive carrier or phage in the co-presence of OCIF or OBM with a change of concentration. For example, screening of a peptide agonist of low molecular weight having an EPO-like activity from a varied peptide library with an erythropoietin (EPO which is a hematopoietic hormone) receptor, analysis of a three-dimensional structure thereof, and the production of substance (agonist) of low-molecular-weight having an EPO activity through synthesis of organic chemical compounds based on the three-dimensional structure have already been succeeded (Nicholas et al.: Science, 273, 458 to 463, 1996).

Furthermore, the inventors have found that a protein binding OCIF is specifically expressed on an osteoblast-like stroma cell line, ST2, which was cultured in the presence of bone resorption factors such as active-form vitamin D₃ and parathyroid hormone (PTH), using osteoclastogenesis inhibitory factor (OCIF). Moreover, the inventors have found that the protein, which is associated with differentiation of immature osteoclast precursor cells to osteoclasts and maturation thereof, has a biological activity as a factor which supports or promotes so-called differentiation and maturation of osteoclast. After purification of the protein, the physicochemical properties and biological activity of the protein were examined. The inventors have compared the

physicochemical properties and biological activity of the recombinant protein OBM by expressing the DNA of the present invention with those of a purified natural-type protein which specifically binds OCIF in order to clarify differences
5 between them. As a result, they have found that (1) each of both proteins is a membrane-bound protein and specifically binds OCIF; (2) they shared a molecular weight of about 40,000 as measured by SDS-PAGE; and (3) they have an apparent molecular weight of about 90,000 to 110,000 when crosslinked
10 with a monomer-type OCIF, which indicates that they have very similar physicochemical properties. An activity to support or promote differentiation and maturation of osteoclast was also shared by them as well. Therefore, the possibility that both proteins are identical was suggested. Furthermore, an anti-
15 OBM rabbit polyclonal antibody prepared with the protein (recombinant OBM), which was genetically expressed with the DNA of the present invention and then purified, has cross-reactivity to the purified natural-type protein obtained by the above method and specifically inhibited the binding
20 between said natural-type protein and OCIF, just as it inhibits specific binding between OBM and OCIF. From these results, it is obvious that the recombinant protein OBM expressed with the DNA of the present is identical to the natural-type protein which specifically binds OCIF.

25 Furthermore, for isolating a gene (cDNA) that encodes a human-derived OCIF binding protein molecule (hereinafter referred to as "human OBM") which specifically binds OCIF and has an activity to support or promote differentiation of mouse spleen cells to osteoclasts and maturation, just as the
30 natural-type or the recombinant mouse OBM does, the inventors have carried out a polymerase chain reaction (PCR) using primers prepared based on the above mouse OBM cDNA and human lymph node-derived cDNA as a template. Thus, the inventors have screened said cDNA library with the obtained human OBM
35 cDNA fragment. As a result, they have succeeded in isolation of the cDNA which encodes the human-derived protein which specifically binds OCIF (human OBM) and determination of the Nucleotide Sequence of said cDNA. They have found that human OBM encoded by the cDNA strongly and specifically binds OCIF

on a cell membrane, and has a biological activity to support or promote differentiation of mouse spleen cells to osteoclasts and maturation thereof, just as mouse OBM does. That is, there are another objects of the present invention to provide DNA which encodes human OBM which is a novel human-derived protein which binds osteoclastogenesis inhibitory factor OCIF; a protein having an amino acid sequence encoded by the DNA; a method for genetically producing a protein which specifically binds OCIF and has an activity to support or promote differentiation of mouse spleen cells to osteoclasts and maturation thereof by use of the DNA,;an agent for treating bone metabolism abnormality comprising the protein; a method for screening a substance which controls expression of human OBM; a method for screening a substance which binds human OBM and inhibits or modifies an effect thereof; a method for screening a receptor which binds human OBM and transmits an effect thereof; and a pharmaceutical composition comprising a substance obtained as a result of these method for screening.

The present invention relates to DNA which encodes human OBM, a novel human protein, which specifically binds OCIF and has a biological activity to support or promote differentiation and maturation of osteoclasts; a protein having an amino acid sequence encoded by the DNA; a method for genetically producing a protein which specifically binds OCIF and has an activity to support or promote differentiation and maturation of osteoclasts with the DNA; and an agent for treating bone metabolism abnormality comprising the protein. Furthermore, the present invention also relates to a method for screening a substance which controls expression of human OBM; a method for screening a substance which binds human OBM and inhibits or modifies an effect thereof; a method for screening a receptor which binds human OBM and transmit a biological activity of OBM; a pharmaceutical composition comprising a substance obtained as a result of these method for screening; an antibody to the human-derived OCIF binding protein; and, an agent for preventing and/or treating bone metabolism abnormality using the antibody.

The novel human-derived OCIF binding protein molecule,

human OBM, encoded by the DNA of the present invention shows the following physicochemical properties and biological activity. That is,

(a) human OBM specifically binds to osteoclastogenesis

5 inhibitory factor (OCIF) (WO96/26217);

(b) human OBM shows a molecular weight of about 40,000 (\pm 5,000) as measured by SDS-PAGE under reducing conditions, and shows an apparent molecular weight of about 90,000 to 110,000 when crosslinked with a monomer-type OCIF; and

10 (c) human OBM has a biological activity to support or promote differentiation and maturation of osteoclast.

The cDNA encoding mouse OBM, mouse-derived OCIF binding protein, useful as a probe for separating and identifying the cDNA which encodes human OBM of the present invention, can be isolated from cDNA library of mouse
15 osteoblast-like stroma cell line, ST2. Furthermore, human osteoclastogenesis inhibitory factor OCIF, required to examine the properties and biological activity of the protein obtained by expressing human OBM cDNA can be isolated from the culture
20 solution of human fibroblast strains IMR-90 in accordance with the method described in WO96/26217, or, can be genetically produced with the DNA encoding it. To examine the properties and biological activity of human OBM, recombinant humOCIF, recombinant mouse OCIF, recombinant rat OCIF and the like can
25 also be used. These recombinant OCIFs can be obtained by incorporating the corresponding cDNAs into expression vectors in accordance with a commonly used method, expressing OCIFs in animal or insect cells such as CHO cells, BHK cells and Namalwa cells, and purifying them.

30 As for a method for isolating the human cDNA which encodes the target protein (cDNA cloning), there are (1) a method comprising the steps of purifying the protein, determining a partial amino acid sequence thereof, and isolating the target cDNA by a hybridization with DNA having a
35 Nucleotide Sequence corresponding to the amino acid sequence as a probe, (2) a method (expression cloning method) comprising the steps of constructing a cDNA library with an expression vector, regardless of whether the amino acid sequence of the target protein is unknown, introducing them

into cells, and screening the presence and absence of the expression of the target protein so as to isolate the target cDNA, and (3) a method of isolating the cDNA which encodes the target human protein by the hybridization or polymerase chain reaction (PCR) method from cDNA library constructed from human cell or tissue, using cDNA which encodes a protein derived from a mammal other than human and having the same properties and biological activity of the human-derived target protein as a probe, based on an assumption that the cDNA which encodes the non-human protein shares high homology with that which encodes the desired corresponding human protein to be cloned.

Based on an assumption that human OBM cDNA shares high homology with the above mouse OBM cDNA, human cell or tissue producing human OBM can be identified by northern hybridization method using the latter (mouse) cDNA as a probe. Human OBM cDNA can be cloned as follows. A human OBM cDNA fragment is obtained through PCR using mouse OBM primers prepared based on the mouse OBM cDNA and cDNA library of cell or tissue which produces human OBM (e.g., a human lymph node) as identified above as primers and a template, respectively; The cDNA library of cell or tissue which produces human OBM as identified above is screened with the human OBM cDNA fragment as a probe; and thus, human OBM cDNA can be obtained. The present invention relates to the obtained DNA that encodes human OBM, a human-derived protein which specifically binds OCIF and has biological activity to support or promote differentiation and maturation of osteoclasts. Since human OBM encoded by the DNA of the present invention is a membrane-bound protein having a transmembrane domain, it can be detected by labeling OCIF and then binding the labeled-OCIF to the surface of an animal cell in which the cDNA of the present invention is expressed. As such a case, OCIF can be labeled by a method which is conventionally used for labeling protein such as labeling with a radioisotope and fluorescence labeling.

The molecular weight of the protein expressed by human OBM cDNA of the present invention is determined by gel filtration chromatography, SDS-PAGE and the like. To determine the molecular weight more accurately, SDS-PAGE is

preferably used, and human OBM is identified as a protein having a molecular weight of about 40,000 ($40,000 \pm 5,000$) under reducing conditions.

5 An example of DNA hybridization under relatively mild conditions in the present invention is that after DNA is transferred to a nylon membrane and fixed in accordance with a commonly used method, the DNA is hybridized with another radio-labeled DNA as a probe in a hybridization buffer at 40 to 70°C for about 2 hours to 1 night and then washed with 0.5 X SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 10 45°C for 10 minutes. More specifically, after DNA is transferred and fixed to a nylon membrane, which is HIBOND N (Amersham Co., Ltd.), in accordance with a conventional method, the DNA is hybridized with another ³²P-labeled DNA as a 15 probe in Rapid Hybridization Buffer (Amersham Co., Ltd.) at 65°C for 2 hours, and then washed with the above 0.5 X SSC at 45°C for 10 minutes.

As a representative in vitro culture system for osteoclastogenesis, a co-culture system of mouse-derived 20 osteoblast-like stroma cell line, ST2, and mouse spleen cells in the presence of an active-form vitamin D₃ or PTH is well known. For promoting osteoclastogenesis in this in vitro culture system, both the interaction between a osteoblast-like stroma cell and a spleen cell through their binding, and the 25 presence of bone resorption factors such as active-form vitamin D₃ and PTH are essential. In this in vitro culture system, a recombinant COS cell strain, resulting from the expression of the cDNA of the present invention thereon, has obtained an ability to support osteoclast formation from 30 spleen cell, just like the osteoblast-like stroma cell line ST2, while COS cell (a monkey kidney-derived cell line) does not an ability to support osteoclast formation in the absence of said bone resorption factors. Furthermore, since the cDNA of the present invention encodes a membrane-bound protein, the 35 protein can be expressed as a secretory-type or solubilized-type protein after removing the fragment which encodes the membrane binding domain thereof. It has also been confirmed that osteoclastogenesis was promoted simply by adding the secretory-type human OBM to the above in vitro culture system

in the absence of said bone resorption factors. From these results, human OBM encoded by the cDNA of the present invention is identified as a factor involved in differentiation and maturation of osteoclasts.

5 Recombinant human OBM can be produced by inserting the cDNA of the present invention into an expression vector so as to prepare a plasmid for expressing human OBM and then introducing and expressing the plasmid in various cells and strains. COS-7, CHO and Namalwa cells and the like can be
10 used as mammalian host cells suitable for expression, and E. coli and the like can be used as bacterial host cells. In those cases, recombinant human OBM can be expressed as a membrane-bound protein by using the full length of DNA, or, as a secretory-type or solubilized-type protein by removing a
15 region which encodes a membrane binding domain. Thus produced recombinant human OBM can be purified efficiently in combination of conventionally used methods for purifying protein such as affinity chromatography using an OCIF-immobilized column, ion exchange chromatography, gel
20 filtration chromatography and the like. Thus obtained human OBM of the present invention is useful, due to its activity, as a medicament, e.g., as an agent for treating bone metabolism abnormality such as osteopetrosis, or as an experimental and diagnostic reagent.

25 The human OBM protein encoded by the cDNA of the present invention enables (1) screening of a substance which controls expression of human OBM, (2) screening of a substance which specifically binds human OBM and inhibits or modifies the biological activity of human OBM, (3) screening of a human
30 protein (human OBM receptor) which exists in a precursor cell of human osteoclast and transmits the biological activity of human OBM, as well as development of antagonist and agonist using this human OBM receptor. In combinatorial chemistry using the above human OBM or human OBM receptor, a peptide
35 library, which is employed to identification of an antagonist or agonist, can be prepared in accordance with the same method using the mouse OBM. After screening the peptide library by said method in which human OBM is used instead of mouse OBM, a specific peptide having very high affinity can be obtained.

Furthermore, for measurement of OBM, a highly useful protein described above, it is necessary to obtain an antibody which specifically recognises OBM and establish an enzyme immunoassay using it. However, no antibody useful for measurement of OBM has been available. Moreover, an anti-OBM/sOBM antibody which neutralizes the biological activity of OBM or sOBM is assumed to suppress an activity of OBM or sOBM to promote osteoclast formation, and expected to be developed as an agent for treating bone metabolism abnormality.

However, such an antibody has not been available.

Under that circumstance, the inventors have made intensive studies and have found antibodies which recognize both of the following antigens, i.e., a membrane-bound protein (OCIF binding molecule; OBM) which specifically binds osteoclastogenesis inhibitory factor (OCIF), and, soluble-type OBM (sOBM) lacking the membrane binding domain (anti-OBM/sOBM antibody). Therefore, an object of the present invention is to provide an antibody which recognizes both of the following antigens, i.e., a membrane-bound protein OBM which specifically bind to osteoclastogenesis inhibitory factor OCIF and a soluble OBM (sOBM) lacking the membrane binding domain (anti-OBM/sOBM antibody); a method for production thereof; a method for measuring OBM and the sOBM by use of said antibody; and an agent for preventing and/or treating bone metabolism abnormality which comprises said antibody as an active ingredient.

The present invention relates to an antibody which recognizes both of the following antigens, i.e., a membrane-bound protein (OCIF binding molecule; OBM) which specifically binds osteoclastogenesis inhibitory factor (OCIF) and soluble-type OBM (sOBM) lacking the membrane binding domain (anti-OBM/sOBM antibody); a method for production thereof; a method for measuring OBM and the sOBM by use of said antibody; and a pharmaceutical composition comprising said antibody as an active ingredient, particularly, an agent for preventing and/or treating bone metabolism abnormality.

An antibody of the present invention is an antibody which has an activity to neutralize osteoclastogenesis promoting activity, which is a biological activity that OBM

and sOBM have, said antibody has any of the following properties:

- a) a polyclonal antibody which recognizes both mouse OBM and mouse sOBM antigens (anti-mouse OBM/sOBM polyclonal antibody),
- 5 b) a polyclonal antibody which recognizes both human OBM and human sOBM antigens (anti-human OBM/sOBM polyclonal antibody),
- c) a monoclonal antibody which recognizes both mouse OBM and mouse sOBM antigens (anti-mouse OBM/sOBM polyclonal antibody),
- d) a monoclonal antibody which recognizes both human OBM and
- 10 human sOBM antigens (anti-human OBM/sOBM polyclonal antibody), and
- e) an anti-human OBM/sOBM monoclonal antibody which has crossreactivity to both mouse OBM and mouse sOBM antigens.

The polyclonal antibody which recognizes both mouse

15 OBM and mouse sOBM antigens (hereinafter referred to as "anti-mouse OBM/sOBM polyclonal antibody") and the polyclonal antibody which recognizes both human OBM and human sOBM antigens (hereinafter referred to as "anti-human OBM/sOBM polyclonal antibody") can be obtained by the following means.

20 A purified mouse OBM as an antigen for immunization can be obtained in accordance with the above method. That is, natural-type mouse OBM can be obtained by treating a mouse osteoblast-like stroma cell line, ST2, with active-form vitamin D₃ and subsequently purifying from the cell membrane of

25 said cell by means of the OCIF-immobilized column and gel filtration chromatography. Alternatively, after incorporating the above mouse OBM cDNA (SEQ ID NO. 15 of Sequence Listing) or human OBM cDNA (SEQ ID NO. 12 of Sequence Listing) into an expression vector, expressing an OBM in an animal or insect

30 cell such as a CHO cell, a BHK cell, Namalwa or a COS-7 cell or E. coli and then purifying by the same method as described above, recombinant mouse OBM (SEQ ID NO. 1 of Sequence Listing) or recombinant human OBM (SEQ ID NO. 11 of Sequence Listing) can be obtained, and these may also be used as

35 antigens for immunization. At this time, it takes tremendous efforts to highly purify a large quantity of mouse OBM or human OBM, a membrane-bound protein (OBM). On the other hand, it has been confirmed that there is no difference in an activity to promote differentiation and maturation of

osteoclasts between OBM, a membrane-bound protein, and soluble-type OBM (sOBM) which is a solubilized protein obtained by deleting the membrane binding domain of OBM, as described above. Accordingly, taking into account expression and high purification of mouse sOBM and human sOBM are relatively easy, these sOBMs, solubilized proteins, may be used as antigens for immunization. Mouse sOBM (SEQ ID NO. 16 of Sequence Listing) and Human sOBM (SEQ ID NO. 17 of Sequence Listing) can be obtained by adding a Nucleotide Sequence which encodes a known signal sequence derived from other secretory-type protein to 5' upstream to mouse sOBM cDNA (SEQ ID NO. 18 of Sequence Listing) or human sOBM cDNA (SEQ ID NO. 19 of Sequence Listing), incorporating the cDNA into an expression vector in accordance with the same gene engineering method as described above, expressing the protein in a variety of animal cells, insect cells or E. coli as a host, and then purifying. Thus obtained antigen for immunization is dissolved in a phosphate buffered saline solution (PBS) and, if necessary, mixed with an equal volume of Freund's complete adjuvant and emulsified. Then, an animal is immunized with the emulsion through a few times of subcutaneous administration with one-week interval each. The antibody titer is measured. When the value reaches maximum, booster administration is performed. On the 10th day from the booster administration, all the blood was collected. The obtained antiserum is fractionated and precipitated with ammonium sulfate, and the globulin fraction is purified with an anion exchange chromatography, or, the antiserum is diluted twice with Binding Buffer (Biorad Co., Ltd.) and the diluted antiserum is purified by protein A or protein G sepharose column chromatography. Thereby, the desired anti-mouse or anti-human OBM/sOBM polyclonal antibody can be obtained.

The monoclonal antibody of the present invention can be obtained by the following method. That is, as an antigen for immunization required to prepare the monoclonal antibody, a natural-type mouse OBM, recombinant mouse or human OBM, or, recombinant mouse or human sOBM can be used, as used in preparation of the above polyclonal antibody. Lymphocyte derived from immunized mammal with each antigen or that

obtained by in vitro method are fused with a myeloma cell line, and hybridomas is prepared, in accordance with a conventional method. From the culture of this hybridoma, a hybridoma producing an antibody which recognizes each antigen is selected by solid-phase ELISA, using each highly purified antigen. The obtained hybridoma is cloned, and thus obtained stable antibody-producing hybridoma is cultured, the target antibody can be obtained therefrom. For preparation of the hybridoma immunizing a mammal, a small animal such as mouse or rat is commonly used. To immunize the animal, a method comprising the following steps is conventionally; diluting the antigen with an appropriate solvent such as physiological saline solution to an appropriate concentration and then administering the solution and, if necessary, co-administering Freund's complete adjuvant, into vein(i.v.) or abdominal cavity(i.p.), about 3 or 4 times in total with 1 to 2-week interval each. The immunized animal is dissected on the 3rd day after the last immunization, and spleen cells are obtained from the isolate spleen and used as immunocytes (immunized cells). Illustrative examples of mouse-derived myeloma for cell fusion with the immunocytes include p3/x63-Ag8, p3-U1, NS-1, MPC-11, SP-2/0, F0, P3x63 Ag8. 653 and S194. Furthermore, illustrative examples of rat-derived cells include cell lines such as R-210. For producing human antibody, human B lymphocyte cells are immunized in vitro and fused with human myeloma cells or cell line transformed by EB virus. Fusion of an immunized cell with a myeloma cell line is performed according to a known method such as that of Kohler and Milstein et al. (Kohler et al.: Nature 256, 495 to 497, 1975) , while electric pulse method using an electric pulse may also be used. Immunized lymphocyte cells and myeloma cell line are mixed together at a ratio conventionally used, and fused in common bovine fetal serum (FCS)-free medium for cell culture in which polyethylene glycol is added. Then, culture is carried out in FCS-containing HAT selective medium so as to select a fused cell (hybridoma). Then, a hybridoma producing an antibody is selected by a commonly used method for detecting antibody such as ELISA, plaque method, ouchterlony method or condensation method. Thereafter, a

hybridoma is established. The established hybridoma can be subcultured by a common method for culture and can be stored in a frozen state if necessary. The hybridoma may be cultured in accordance with a conventionally used method or

5 transplanted in the abdominal cavity of the mammal. The antibody can be collected from the resulting culture solution or ascites, respectively. The antibody in the culture solution or ascites can be purified by a commonly used method such as salting-out method, ion exchange chromatography, gel
10 filtration chromatography, or, protein A or protein G affinity chromatography. Almost all the monoclonal antibodies obtained by the above-described method using sOBM as an antigen are an antibody which can specifically recognize not only sOBM but also OBM (hereinafter referred to as "anti-OBM/sOBM monoclonal
15 antibody"). These antibodies can be used for measurements of OBM and sOBM. After these antibodies are labeled with a radioisotope or an enzyme and thus employed to measurement systems known such as as radioimmunoassay (RIA) and enzyme immunoassay (EIA), an amount of OBM and sOBM can be measured
20 thereby. By use of these measurement systems, an amount of sOBM in a living sample such as blood or urine can be measured with ease and with high sensitivity. Furthermore, by use of these antibodies, an amount of OBM bound to the surface of a tissue or cell can be measured through binding assay or the
25 like with ease and with high sensitivity.

When the obtained antibody is used as a medicament for humans, it is desirable, in consideration of a problem of antigenicity, that a human-type anti-human OBM/sOBM antibody is prepared. The human-type anti-human OBM/sOBM antibody can
30 be prepared by the following methods. That is, (1) human lymphocyte cells extracted from human peripheral blood or the spleen are sensitized in vitro with human OBM or human sOBM as an antigen in the presence of IL-4, and then the sensitized human lymphocyte cells are fused with K₆H₆/B₅ (ATCC CRL1823),
35 which is a hetero hybridoma of mouse and human, thereby, a hybridoma producing the desired antibody is screened. An antibody produced from the obtained hybridoma is a human-type anti-human OBM/sOBM monoclonal antibody. Among these antibodies, an antibody which neutralizes the activity of

human OBM/sOBM is selected. However, it is usually difficult to obtain an antibody having high affinity for an antigen through such a method that of sensitizing human lymphocyte cells in vitro. Therefore, for obtaining a monoclonal antibody having high affinity for human OBM and sOBM, it is necessary to modify an anti-human OBM/sOBM monoclonal antibody with low affinity as described above to be that with high affinity. A random mutation is introduced into a CDR region (CDR-3 in particular) of said human-type anti-human OBM/sOBM monoclonal antibody with low affinity which a neutralizing antibody obtained as described above; this is expressed with phage; phage which strongly binds human OBM/sOBM as the antigen is selected by phage display method using a plate in which human OBM/sOBM is immobilized; the phage is allowed to proliferate in *E. coli*; and the deduced amino acid sequence of the CDR having high affinity is determined based on the Nucleotide Sequence thereof. The obtained gene which encodes the human-type anti-human OBM/sOBM monoclonal antibody is incorporated and expressed in a conventionally used expression vector for mammalian cell, and then human-type anti-human OBM/sOBM monoclonal antibodies can be obtained. Among them, the desired human-type anti-human OBM/sOBM monoclonal antibody which neutralizes the biological activity of human OBM/sOBM and has high affinity thereto can be selected. Furthermore, (2) using a Balb/c mouse, an anti-human OBM/sOBM mouse monoclonal antibody is prepared according to a conventionally used method (Koehler et al., *Nature* 256, 495 to 497, 1975) as in the present invention, and a monoclonal antibody which neutralizes the biological activity of human OBM/sOBM and has high affinity thereto is selected. By CDR grafting method (Winter and Milstein: *Nature* 349, 293 to 299, 1991), that is a method in which a CDR region (CDR-1, 2 and 3) of the anti-human OBM/sOBM mouse monoclonal antibody with high affinity are transplanted into the CDR regions of human IgG, a humanized antibody can be obtained. Moreover, (3) human peripheral blood lymphocyte cells are transplanted into a severe combined immune deficiency (SCID) mouse. Thus transplanted SCID mouse produces a human antibody (Mosier D. E. et al.: *Nature* 335, 256 to 259, 1988; Duchosal M. A. et

al.: Nature 355, 258 to 262, 1992). The cells are sensitized with human OBM or sOBM as an antigen and screened.

Thereafter, a lymphocyte cell which produce a human-type monoclonal antibody specific to human OBM/sOBM can be

5 extracted from the mouse. Then, as in the case of the above method for preparing a human-type antibody (1), the obtained lymphocyte cells are fused with K₆H₆/B₅ (ATCC CRL1823), a hetero hybridoma of mouse and human, and then the obtained hybridomas are screened. Then, a hybridoma which produces the target
10 human-type monoclonal antibody can be obtained. By culturing the thus obtained hybridoma, the target human-type monoclonal antibody can be produced in large quantity. After purifying them in the same manner as described above, large amount of pure products thereof can be obtained. Furthermore, a
15 recombinant human-type monoclonal antibody can be produced in large quantity by constructing a cDNA library from said hybridoma which produces the target human-type monoclonal antibody, cloning cDNA which encodes the target human-type monoclonal antibody, incorporating said cDNA into an
20 appropriate expression vector by gene engineering, and expressing the antibody in a variety of animal cells, insect cells or E. coli as a host. After purification of the antibody from said culture according to the method as described above, a large amount of pure human-type monoclonal antibody can be
25 obtained.

Among the anti-OBM/sOBM monoclonal antibodies obtained by the above method, moreover, an antibody which neutralizes the biological activity of OBM/sOBM can be obtained. These antibodies which neutralize the biological activity of
30 OBM/sOBM are expected as medicaments, particularly agents for preventing and/or treating bone metabolism abnormality, since they can inhibit the biological action (an activity to promote osteoclast formation) of OBM/sOBM in a living body. The activity of the anti-OBM/sOBM antibody to neutralize the
35 biological activity of OBM or sOBM can be determined as an activity to inhibit osteoclast formation in an in vitro system for examining osteoclast formation. As in vitro assay systems, the following three methods can be used. That is, as in vitro culture systems for examining osteoclastogenesis, (1)

a co-culture system of a mouse osteoblast-like stroma cell line, ST2, and mouse spleen cell in the presence of active-form vitamin D₃ and dexamethasone, (2) a co-culture system in which an OBM is expressed on a monkey kidney cell line, COS-7, cell and fixed with formaldehyde, and then mouse spleen cell is cultured on the cell in the presence of M-CSF, (3) a system of culturing mouse spleen cell in the presence of recombinant sOBM and M-CSF, however, other systems can be also used. When an anti-OBM/sOBM antibody is added to such a culture system in various concentration and its effect on osteoclastogenesis is examined, an activity of the anti-OBM/sOBM antibody to inhibit osteoclastogenesis can be measured. Also, the activity of the anti-OBM/sOBM antibody to inhibit osteoclastogenesis can be determined as an activity to suppress bone resorption in vivo using an experimental animal. That is, there is an animal model, overiectomized model, in which osteoclastogenesis is increased. An anti-OBM/sOBM antibody is administered to such a kind of experimental animal, and an activity to inhibit bone resorption (an activity to reinforce bone mineral density) is measured. Thereby, an activity of the anti-OBM/sOBM antibody to inhibit osteoclastogenesis can be determined.

The obtained antibody which neutralizes the biological activity of OBM/sOBM is useful as medicaments, particularly as a pharmaceutical composition for preventing and/or treating bone metabolism abnormality, or as an antibody used in immunological diagnosis of such a disease. The antibody of the present invention can be prepared in a formulation, and administered orally or parenterally. A formulation comprising the antibody of the present invention is administered safely to human or animal as a pharmaceutical composition comprising the antibody which recognizes OBM and/or sOBM as an active ingredient. Illustrative examples of the formulation of the pharmaceutical composition include injectable solutions such as drip, suppository, nasogastric agent, sublingual agent and transdermal agent. Since the monoclonal antibody has high molecular weight, its adsorption to glass containers such as vial and syringe tube is significant. Furthermore, the antibody is unstable and easily inactivated due to various physicochemical factors such as heat, pH and humidity. Thus,

to formulate stably the antibody, stabilizer, pH adjuster, buffer, solubilizing agent, surfactant and the like are added thereto. Illustrative examples of the stabilizer include amino acids such as glycine and alanine, saccharides such as dextran 40 and mannose, and sugar alcohols such as sorbitol, mannitol and xylitol. These may be used in combination of two or more. These stabilizer are preferably added in an amount which is 0.01 to 100 times, particularly 0.1 to 10 times as much as the weight of the antibody. By addition of these stabilizer, the storage stability of liquid formulation or freeze-dried formulation can be improved. Illustrative examples of buffer include phosphate buffer and citric acid buffer. The buffer adjusts the pH of an aqueous formulation or a reconstituted solution of freeze-dried formulation, and thereby contributes to the stability and solubility of the antibody therein. The amount of the buffer is preferably, for example, 1 to 10 mM in an aqueous formulation or a reconstituted solution of freeze-dried formulation. The surfactant is preferably Polysorbate 20, Pluronic F-68 and polyethylene glycol, particularly preferably Polysorbate 20. These may be used in combination of two or more. A protein having high molecular weight like antibody is liable to adsorb to glass or resin, which a container is made of. However, by addition of a surfactant to , adsorption of the antibody to a container in an aqueous formulation or a reconstituted solution of freeze-dried formulation can be prevented. The surfactant is preferably added in an amount of 0.001 to 1.0% of the weight of an aqueous formulation or a reconstituted solution of freeze-dried formulation. The formulation comprising the antibody of the present invention can be prepared by addition of the stabilizer, buffer and adsorption-preventing agent as described above. Particularly, when it is used as an injectable formulation for medical applications or treating animals, acceptable osmotic pressure ratio is preferably 1 or 2. The osmotic pressure ratio can be adjusted by increasing or decreasing sodium chloride in formulation. The content of the antibody in the formulation can be adjusted appropriately, depended on disease to be treated with said formulation, administration route and the like. The dose of the human-type

antibody administered to human depends on the affinity of the antibody to human OBM/sOBM, that is, dissociation constant (K_d value) of the antibody to human OBM/sOBM. The higher the affinity is (or the lower the K_d value is), the smaller dose is required to exhibit medicinal benefits. Furthermore, since half-life time of human-type antibody in human blood is about 20 days, the human-type antibody can be administered to human in a dose of about 0.1 to 100 mg/kg at least once within 1 to 30 days, for example.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the results of SDS-PAGE of mouse OBM protein of Example 3 of the present invention, wherein:

(A): Lane 1: molecular weight marker,

Lane 2: partially purified fraction eluted with Gly-HCl (pH: 2.0), which was derived from ST2 cells cultured in the presence of active-form vitamin D₃ and dexamethasone,

Lane 3: partially purified fraction eluted with Gly-HCl (pH: 2.0), which was derived from ST2 cells cultured in the absence of active-form vitamin D₃ and dexamethasone,

(B): Lane 1: molecular weight marker,

Lane 2: mouse OBM protein (Example 3) of the present invention purified with reversed phase high performance liquid chromatography.

Fig. 2 shows the results of binding experiment of the ¹²⁵I-labeled OCIF to an osteoblast-like stroma cell, ST2, in Example 4.

Fig. 3 shows the osteoclastogenesis-supporting activity of osteoblast-like stroma cell, ST2, with different passage numbers, in Example 5(1), wherein:

1: osteoclastogenesis-supporting activity of ST2 cell with a passage number of around 10's,

2: osteoclastogenesis-supporting activity of ST2 cell with a passage number of around 40's.

Fig. 4 shows a change in expression of the protein of the present invention on an osteoblast-like stroma cell membrane, said cell was cultured in the presence of an active-form vitamin D₃ and dexamethasone, with passage of time, in Example 5(2).

Fig. 5 shows a change in osteoclastogenesis in the co-culture system, with passage of time, of Example 5(2).

Fig. 6 shows osteoclastogenesis-inhibiting effects when OCIF was treated only during various culturing periods in the co-culture period of Example 5(3).

Fig. 7 shows the results of crosslinking experiment of the ^{125}I -labeled OCIF with the protein of the present invention, in Example 6, wherein:

Lane 1: ^{125}I -labeled OCIF-CDD1,

Lane 2: sample resulting from crosslinking of ^{125}I -labeled OCIF-CDD1 with an ST2 cell,

Lane 3: sample resulting from crosslinking with an ST2 cell in the presence of 400-fold higher concentration of unlabeled OCIF than that of ^{125}I -OCIF.

Fig. 8 shows the results of SDS-PAGE in Example 9, wherein:

Lane 1: precipitate resulting from immuno-precipitation of the protein of COS-7 cells transfected with pOBM291 without OCIF,

Lane 2: precipitate resulting from immuno-precipitation of the protein of COS-7 cells transfected with pOBM291 with OCIF.

Fig. 9 shows the results of binding experiment of ^{125}I -labeled OCIF to COS-7 cells transfected with pOBM291 in Example 10, wherein:

Lanes 1 and 2: amount of ^{125}I -labeled OCIF bound to COS-7 cells transfected with pOBM291,

Lanes 3 and 4: amount of ^{125}I -labeled OCIF bound to COS-7 cells transfected with pOBM291 in the presence of 400-fold higher concentration of unlabeled OCIF than that of ^{125}I -OCIF.

Fig. 10 shows the results of crosslinking experiment using ^{125}I -labeled OCIF in Example 11, wherein:

Lane 1: ^{125}I -labeled OCIF,

Lane 2: sample resulting from crosslinking of ^{125}I -labeled OCIF with COS-7 cells transfected with pOBM291,

Lane 3: sample resulting from crosslinking of ^{125}I -labeled OCIF with COS-7 cells transfected with pOBM291 in the presence of 400-fold higher concentration of unlabeled OCIF than that of ^{125}I -OCIF.

Fig. 11 shows the results of northern blot in Example 12, wherein:

Lane 1: RNA derived from ST2 cells cultured in the absence of vitamin D and dexamethasone,

Lane 2: RNA derived from ST2 cells cultured in the presence of vitamin D and dexamethasone.

5 Fig. 12 shows OCIF binding ability of a protein in the conditioned medium when the concentration of OCIF was varied in Example 13-(2), wherein:

○: pCEP4,

●: pCEP sOBM.

10 Fig. 13 shows the OCIF binding ability of the protein in the conditioned medium when the amount of the conditioned medium was varied in Example 13-(2), wherein:

○: pCEP4,

●: pCEP sOBM.

15 Fig. 14 shows the results of SDS-PAGE of a fusion protein of thioredoxin and mouse OBM expressed in E. coli, in Example 14-(2), wherein:

Lane 1: molecular weight marker,

Lane 2: soluble protein fraction derived from GI724/pTrxFus,

20 Lane 3: soluble protein fraction derived from GI724/pTrxOBM25.

 Fig. 15 shows OCIF binding abilities when the amount of the soluble protein fractions were varied in Example 14-(3), wherein:

□: GI724/pTrxFus,

25 ○: GI724/pTrxOBM25.

 Fig. 16 shows the OCIF binding abilities of the soluble protein fractions (1%) when the concentration of OCIF was varied in Example 14-(3), wherein:

□: GI724/pTrxFus,

30 ○: GI724/pTrxOBM25.

 Fig. 17 shows the results of inhibition of the specific binding of the mouse protein obtained by expressing the mouse OBM cDNA of the present invention and purifying (mouse OBM) and the purified natural-type OCIF binding protein to OCIF, by an anti-mouse OBM rabbit antibody, wherein:

1: purified recombinant OBM treated with an antibody + ¹²⁵I-OCIF,

2: the purified natural-type protein treated with an antibody + ¹²⁵I-OCIF,

3: purified recombinant OBM untreated with an antibody + ^{125}I -OCIF,

4: the purified natural-type protein untreated with an antibody + ^{125}I -OCIF,

5 5: 3 + unlabeled OCIF (400-fold higher concentration than that of ^{125}I -OCIF),

6: 4 + unlabeled OCIF (400-fold higher concentration than that of ^{125}I -OCIF).

10 Fig. 18 shows the results of SDS-PAGE of human OBM protein expressed by the cDNA of the present invention, wherein:

Lane 1: molecular weight marker,

15 Lane 2: precipitate resulting from immuno-precipitation of the protein derived from COS-7 cells transfected with an expression vector phOBM containing the cDNA of the present invention by an anti-OCIF rabbit polyclonal antibody without OCIF,

20 Lane 3: precipitate resulting from immuno-precipitation of the protein derived from COS-7 cells transfected with an expression vector phOBM containing the cDNA of the present invention by an anti-OCIF rabbit polyclonal antibody with OCIF.

25 Fig. 19 shows the results of binding experiment of OCIF to COS-7 cells transfected with an expression vector phOBM containing the cDNA of the present invention, wherein:
Lane 1: COS-7 cells transfected with phOBM + ^{125}I -OCIF,
Lane 2: COS-7 cells transfected with phOBM + ^{125}I -OCIF + 400-fold higher concentration of unlabeled OCIF than that of ^{125}I -OCIF.

30 Fig. 20 shows the results of crosslinking experiment of human OBM protein encoded by the cDNA of the present invention with ^{125}I -OCIF (monomer type), wherein:

Lane 1: ^{125}I -OCIF,

35 Lane 2: sample resulting from crosslinking of ^{125}I -OCIF with a protein on the membrane of COS-7 cells transfected with phOBM,
Lane 3: sample resulting from crosslinking of ^{125}I -OCIF with a protein on the membrane of COS-7 cells transfected with phOBM in the presence of 400-fold higher concentration of unlabeled OCIF than that of ^{125}I -OCIF.

Fig. 21 shows the OCIF binding ability of a protein (secretory-type human OBM) in the conditioned medium when the concentration of OCIF was varied in Example 23-(2), wherein:
○: conditioned medium of 293-EBNA cell transfected with pCEP4
5 vector not containing cDNA which encodes the secretory-type human OBM,

●: conditioned medium of 293-EBNA cell transfected with pCEPshOBM expression vector containing cDNA which encodes the secretory-type human OBM.

10 Fig. 22 shows the OCIF binding ability of the protein (secretory-type human OBM) in the conditioned medium when the amount of the conditioned medium to be added was varied while the concentration of OCIF was kept constant, in Example 23-(2), wherein:

15 ○: conditioned medium of 293-EBNA cells transfected with pCEP4 vector not containing cDNA which encodes the secretory-type human OBM,

●: conditioned medium of 293-EBNA cells transfected with pCEPshOBM expression vector containing cDNA which encodes the
20 secretory-type human OBM.

Fig. 23 shows the results of SDS-PAGE of a fusion protein of thioredoxin and human OBM, expressed in E. coli, wherein:

Lane 1: molecular weight marker,

25 Lane 2: soluble protein fraction derived from E. coli GI724/pTrxFus,

Lane 3: soluble protein fraction derived from E. coli GI724/pTrxOBM.

Fig. 24 shows the ability of a fusion protein to bind
30 OCIF when the amount of soluble protein fraction containing the fused protein of thioredoxin and human OBM expressed in E. coli was varied, in Example 24-(3), wherein:

○: soluble protein fraction derived from E. coli GI724/pTrxFus,

35 ●: soluble protein fraction derived from E. coli GI724/pTrxshOBM.

Fig. 25 shows the ability of the fusion protein of thioredoxin and human OBM in soluble protein fraction of E. coli to bind OCIF when the concentration of OCIF was varied,

in Example 24-(3), wherein:

○: soluble protein fraction derived from E. coli GI724/pTrxFus

●: soluble protein fraction derived from E. coli
GI724/pTrxshOBM.

5 Fig. 26 shows the results of measurement of human OBM
and human sOBM by sandwich ELISA using an anti-human OBM/sOBM
rabbit polyclonal antibody of the present invention, wherein:
□: human OBM,
●: human sOBM.

10 Fig. 27 shows the results of measurement of human OBM
and human sOBM by sandwich ELISA using an anti-human OBM/sOBM
monoclonal antibody of the present invention, wherein:
□: human OBM,
●: human sOBM.

15 Fig. 28 shows the results of measurement of mouse OBM
and mouse sOBM by sandwich ELISA using an anti-human OBM/sOBM
monoclonal antibody of the present invention, said antibody
has cross-reactivity to both mouse OBM and mouse sOBM,
wherein:

20 □: mouse OBM,
●: mouse sOBM.

Fig. 29 shows an activity of a fusion protein of
thioredoxin and mouse OBM to promote the formation of human
osteoclast-like cell

25 Fig. 30 shows suppression of vitamin D₃-stimulated
bone resorption by an anti-OBM/sOBM antibody.

Fig. 31 shows suppression of prostaglandin E₂ (PGE₂)-
stimulated bone resorption by an anti-OBM/sOBM antibody.

30 Fig. 32 shows suppression of parathyroid hormone
(PTH)-stimulated bone resorption by an anti-OBM/sOBM antibody.

Fig. 33 shows suppression of interleukin 1 α (IL-1)-
stimulated bone resorption by an anti-OBM/sOBM antibody.

BEST MODE FOR PRACTICING THE INVENTION

35 [Examples]

The present invention is explained in more detail with
reference to the following Examples. However, these Examples
are only exemplary and shall not limit the present invention
thereto in any way.

[Example 1]

Production of the Protein of the Present Invention

(1) Large Scale Culture of ST2 Cells

Mouse osteoblast like stroma cell line, ST2, (RIKEN
5 CELL BANK, RCB0224) was cultured with α -MEM medium containing
10% bovine fetal serum. After cultured to become confluent in
a 225-cm² T flask for adherent cells, ST2 cells were treated
with trypsin, stripped from the T flask, washed, and then
transferred to five of 225-cm² T flask. After addition of 60
10 ml of α -MEM medium containing 10^{-8} M active-form vitamin D₃
(Calcitriol), 10^{-7} M dexamethasone and bovine fetal serum, the
resulting cells were cultured in a CO₂ incubator for 7 to 10
days. The cultured ST2 cells were recovered using a cell
scraper and stored at -80°C until use.

15 (2) Preparation of Membrane Fraction and Solubilization of
Membrane-Bound Protein

To ST2 cells (amount: about 12 ml) described in
Example 1-(1) which were cultured with 80 of 225-cm² T flasks,
a 3-fold volume (36 ml) of 10 mM Tris-hydrochloric acid buffer
20 (pH: 7.2) containing protease inhibitors (2 mM APMSFP, 2 mM
EDTA, 2 mM o-phenanthroline, 1 mM leupeptin, 1 μ g/ml pepstatin
A and 100 units/ml aprotinin) were added. After these cells
were vigorously agitated by use of a vortex mixer for 30
seconds, they were left to stand on ice for 10 minutes. Using
25 a homogenizer (DOUNCE TISSUE GRINDER, A syringe, WHEATON
SCIENTIFIC CO., LTD.), these cells were crushed. To these
crushed cell solution, an equal volume (48 ml) of 10 mM Tris-
hydrochloric acid buffer (pH: 7.2) containing the above
protease inhibitors, 0.5 M sucrose, 0.1 M potassium chloride,
30 10 mM magnesium chloride and 2 mM calcium chloride was added,
and thus obtained mixture was agitated, and then centrifuged
at 600Xg at 4°C for 10 minutes. Through this centrifugation,
cell nuclei and uncrushed cells were separated as precipitated
fractions. A supernatant obtained after centrifugation was
35 further centrifuged at 150,000Xg at 4°C for 90 minutes, and
membrane fractions of the ST2 cells were obtained as
precipitated fractions. To the membrane fractions, 8 ml of 10
mM Tris-hydrochloric acid buffer (pH: 7.2) containing the

above protease inhibitors, 150 mM of sodium chloride and 0.1 M sucrose was added, and then 200 μ l of 20% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, sigma Co., Ltd.) was added. The mixture was agitated at 4°C for 2 hours. This solution was centrifuged at 150,000Xg at 4°C for 60 minutes, and the resulting supernatant was obtained as a solubilized membrane fraction.

[Example 2]

Purification of the Protein of the Present Invention

(1) Preparation of OCIF-Immobilized Affinity Column

Isopropanol in a HiTrap NHS-activated column (1 ml, Pharmacia Co., Ltd.) was substituted with 1 mM hydrochloric acid, and 1 ml of 0.2 M NaHCO_3 /0.5 M NaCl (pH: 8.3) solution containing 13.0 mg of recombinant OCIF prepared in accordance with a method described in WO96/26217 was added to the column using a syringe (5 ml, TERUMO CORPORATION). After the column was allowed to undergo a coupling reaction at room temperature for 30 minutes, 3 ml of 0.5 M ethanolamine/0.5 M NaCl (pH: 8.3) and 3 ml of 0.1 M acetic acid/0.5 M NaCl (pH: 4.0) were loaded on the column alternately three times each in total so as to inactivate excessive activated groups. Then, the mobile phase of the column was substituted again with 0.5 M ethanolamine/0.5 M NaCl (pH: 8.3) and then left to stand at room temperature for 1 hour. Thereafter, the resulting column was washed twice with 0.5 M ethanolamine/0.5 M NaCl (pH: 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH: 4.0) and then the mobile phase was substituted with 50 mM Tris/1M NaCl/0.1% CAHPS buffer (pH: 7.5).

(2) Purification of the Protein of the Present Invention by OCIF-Immobilized Affinity Column

Purification of OCIF binding protein was carried out at 4°C unless otherwise stated. The above OCIF-immobilized affinity column was equilibrated with 10 mM Tris-hydrochloric acid buffer (pH: 7.2) containing the protease inhibitors described in Example 1-(2), 0.15 M sodium chloride and 0.5% CHAPS. To this column, about 8 ml of the solubilized membrane fraction described in Example 1-(2) was applied at a flow rate of 0.01 ml/min. The column was washed with the above 10 mM Tris-hydrochloric acid buffer (pH: 7.2) containing the

protease inhibitors, 0.15 M sodium chloride and 0.5% CHAPS at a flow rate of 0.5 ml/min for 100 minutes. Then, the proteins were eluted from the column with 0.1 M glycine-hydrochloric acid buffer (pH: 3.3) containing the above protease inhibitors, 0.2 M sodium chloride and 0.5% CHAPS at a flow rate of 0.1 ml/min for 50 minutes. Similarly, a 0.1 M sodium citrate buffer (pH: 2.0) containing said protease inhibitors, 0.2 M sodium chloride and 0.5% CHAPS was fed to the column at a flow rate of 0.1 ml/min for 50 minutes so as to elute proteins adsorbed to the column. The eluates were fractionated as 0.5 ml/fraction each. The fractions were immediately neutralized by addition of a 2M Tris solution. The fractions (the volume of the eluate was 1.0 to 5.0 ml) eluted with the buffer were concentrated to 50 to 100 μ l using Centricon-10 (Amicon, USA). Aliquots of the concentrated fractions were subfractionated, and after addition of OCIF to the aliquots, they were immunoprecipitated with an ant-OCIF polyclonal antibody. After the precipitated fractions were treated with SDS, they were subjected to SDS-PAGE, and then a fraction (Fr. Nos. 3-10) showing a band of the protein having an activity to specifically bind OCIF was identified as the protein fraction of the present invention.

(3) Purification of the Protein of the Present Invention by Gel Filtration

The OCIF binding protein eluted with 0.1 M glycine-hydrochloric acid buffer (pH3.3) and subsequently 0.1 M sodium citrate buffer (pH2.0) after purification and concentration in accordance with the method described in Example 2-(2) was subjected to a Superose 12 HR10/30 column (Pharmacia Co., Ltd., 1.0 X 30 cm) equilibrated with 10 mM Tris-HCl, 0.5 M NaCl and 0.5% CHAPS (pH: 7.0) and developed using the above equilibration buffer as a mobile phase at a flow rate of 0.5 ml/min, and then fractions of 0.5 ml were collected. The fraction containing the protein of the present invention (Fr. Nos. 27-32) was identified and concentrated by means of Centricon-10 (Amicon) in the same manner as described above.

(4) Purification by Reversed Phase High Performance Liquid Chromatography

OCIF binding protein purified by the above gel

filtration was added to a C₄ column (2.1 X 250 mm, Vydac, USA) equilibrated with 0.1% trifluoroacetic acid (TFA) and 30% acetonitrile. Elution was carried out at a flow rate of 0.2 ml/min with the gradient of acetonitrile concentration of from 30% to 55% for 50 minutes and then of from 55% to 80% for another 10 minutes. The peaks of eluted proteins were detected at 215 nm. The eluted protein of each peak was fractionated, and the peak of the protein of the present invention was identified. Thus, a highly purified protein of the present invention was obtained.

[Example 3]

SDS-PAGE of the Purified Protein of the Present Invention

Firstly, solubilized membrane fraction prepared from ST2 cells which were cultured in the presence or absence of active-form vitamin D₃ was purified with the OCIF-immobilized affinity column as described above, and the purified samples were subjected to SDS-PAGE. As shown in Fig. 1(A), it was revealed that a major protein band of about 30,000 to 40,000 was detected only in the purified sample obtained from the ST2 cells cultured in the presence of active-form vitamin D₃, and that a protein which specifically binds OCIF, i.e., the protein of the present invention, is selectively concentrated and purified with the OCIF-immobilized affinity column. However, in addition to the protein of the present invention, some other bands of proteins which were nonspecifically bound to the carriers, spacers or the like of the OCIF-immobilized column were also detected in both purified samples. These proteins other than the protein of the present invention were removed by gel filtration and C₄ reversed phase chromatography as described above. The SDS-PAGE of the obtained highly purified protein of the present invention is shown in Fig. 1(B). The highly purified protein of the present invention was electrophoretically homogeneous, and the molecular weight thereof was about 30,000 to 40,000.

[Example 4]

Examining the Binding of OCIF to Osteoblast

(1) Preparation of ¹²⁵I-Labeled OCIF

OCIF was ¹²⁵I-labeled by Iodogen method. More specifically, 20 µl of 2.5 mg/ml Iodogen-chloroform solution

was transferred to a 1.5 ml Eppendorf tube, and chloroform was evaporated at 40°C so as to prepare an Iodogen-coated tube. After the tube was washed three times with 400 µl of 0.5 M sodium phosphate buffer (Na-Pi, pH: 7.0), 5 µl of 0.5 M Na-Pi (pH7.0) was added thereto. Immediately after 1.3 µl (18.5 MBq) of Na-¹²⁵I solution (Amersham Co., Ltd., NEZ-033H20) was added to the tube, 10 µl of 1 mg/ml rOCIF solution (monomer type or dimer type) was added. The obtained solution was agitated with a vortex mixer, and then left to stand at room temperature for 30 seconds. The solution was transferred to a tube containing 80 µl of 10 mg/ml potassium iodide and 0.5 M Na-Pi solution (pH7.0), and 5 µl of phosphate buffered saline solution containing 5% bovine serum albumin, and then agitated. This solution was applied to a spin column (1 ml, G-25 fine, Pharmacia Co., Ltd.) equilibrated with a phosphate buffered saline solution containing 0.25% bovine serum albumin and centrifuged at 2,000 rpm for 5 minutes. After adding 400 µl of phosphate buffered saline solution containing 0.25% bovine serum albumin to the fraction eluted from the column and subsequently mixing, 2 µl aliquot was collected, and the radioactivity thereof was measured with a gamma counter. The radiochemical purity of the thus prepared ¹²⁵I-labeled OCIF solution was determined by measuring the radioactivity of a fraction precipitated by 10% TCA. Furthermore, the biological activity as OCIF the ¹²⁵I-labeled OCIF solution was determined in accordance with a method described in WO96/26217. Moreover, the concentration of ¹²⁵I-labeled OCIF was measured by ELISA in the following manner.

(2) Measurement of Concentration of ¹²⁵I-Labeled OCIF by ELISA

100 µl of 50 mM NaHCO₃ (pH: 9.6) in which 2 µg/ml of anti-OCIF rabbit polyclonal antibody described in WO96/26217 was dissolved was added to each well of 96-well immunoplate (MaxiSorp™, Nunc Co., Ltd.) and left to stand at 4°C overnight. After this solution was removed, 300 µl of BLOCKACE (Snow Brand Milk Products Co., Ltd.)/phosphate buffered saline solution (25/75) was added to each well and then left to stand at room temperature for 2 hours. After this solution was

removed, each well was washed three times with phosphate buffered saline solution (P-PBS) containing 0.01% polysorbate 80. Thereafter, 300 μ l of BLOCKACE/phosphate buffered saline solution (25/75) containing 125 I-labeled OCIF sample or standard OCIF was added to each well and left to stand at room temperature for 2 hours. After this solution was removed, each well was washed six times with 200 μ l of P-PBS. Then, 100 μ l of BLOCKACE (Snow Brand Milk Products Co., Ltd.)/phosphate buffered saline solution (25/75) containing peroxidase-labeled anti-OCIF rabbit polyclonal antibody was added to each well and left to stand at room temperature for 2 hours. After this solution was removed, each well was washed six times with 200 μ l of P-PBS. Then, 100 μ l of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well and then left to stand at room temperature for 2 to 3 minutes. Thereafter, 100 μ l of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at 490 nm was measured with a microplate reader. The concentration of the 125 I-labeled OCIF was calculated from a calibration curve made by using standard OCIF.

(3) Examining the Binding of OCIF to Osteoblast or Pancreas cell

A mouse osteoblast like stroma cell line, ST2, or mouse pancreas cell was suspended in α -MEM medium containing 10% bovine fetal serum (FBS) with or without 10^{-8} M active-form vitamin D₃ (Calcitriol) and 10^{-7} M dexamethasone at a concentration of 4×10^4 cell/ml and 2×10^6 cell/ml, respectively. 1 ml of this medium was seeded in a 24 well microplate. After the cells were cultured in a CO₂ incubator for 4 days and washed with α -MEM medium, 200 μ l of medium for binding experiment (α -MEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer and 0.2% NaN₃) further containing 20 ng/ml of the above 125 I-labeled OCIF (monomer type or dimer type) was added to each well. Furthermore, 200 μ g/ml of the medium for binding experiment containing 8 μ g/ml rOCIF (400-fold higher concentration) was added to other wells which were subjected to measurements of nonspecific binding. After the

cells were cultured in a CO₂ incubator for 1 hour, they were washed three times with 1 ml of phosphate buffered saline solution. Since the pancreas cells are floating cells, cells in each well were washed in the 24-well plate with centrifugation. After washing, 500 µl of 0.1 N NaOH solution was added to each well and left to stand at room temperature for 10 minutes. Thereby, the cells were washed, and the amount of RI bound to the cells was measured with a gamma counter.

The ¹²⁵I-labeled OCIF did not bind the cultured pancreas cells, but specifically bound only osteoblast like stroma cell cultured in the presence of active-form vitamin D₃. Thereby, it was revealed that the protein of the present invention was a membrane-bound protein induced on the cell surface of an osteoblast like stroma cell with active-form vitamin D₃ and dexamethasone.

[Example 5]

Biological Activity of the Protein of the Present Invention

(1) Ability of Osteoblast Like Stroma Cell to Support

Osteoclast Formation

The ability of osteoblast to support osteoclast formation was examined by measuring tartaric acid resistant acid phosphatase activity (TRAP activity) of the formed osteoclast. More specifically, the pancreas cells (2 X 10⁵ cells/100 µl/well) and mouse osteoblast like stroma cell line, ST2, (5 X 10³ cells/100 µl/well) derived from a ddy mouse (8 to 12 weeks old) were suspended in α-MEM medium containing 10% bovine fetal serum, 10⁻⁸ M active-form vitamin D₃ and 10⁻⁷ M dexamethasone and seeded in a 96-well plate. After the cells were cultured in a CO₂ incubator for one week, each well was washed with phosphate buffered saline solution. Then, 100 µl of ethanol/acetone (1:1) was further added to the wells and fixed at room temperature for 1 minute. Then, 100 µl of 50 mM citric acid buffer (pH: 4.5) containing 5.5 mM p-nitrophenol phosphate and 10 mM sodium tartrate was added to each well and then allowed to react at room temperature for 15 minutes. After the reaction, 0.1 N NaOH solution was added to each well, and the absorbance at 405 nm was measured with a

microplate reader. Fig. 3 shows the results of examining the abilities of ST2 cells to support osteoclast formation, wherein the passage number of said cells were around 10's or around 40's (after purchased from RIKEN CELL BANK.). From these results, it was revealed that ST2 cell of high passage number had high ability to support osteoclast formation.

(2) Changes with the Passage of Time in Expression of the Protein of the Present Invention on the Membrane of Osteoblast Like Stroma Cell Cultured in the Presence of Active-Form Vitamin D₃ and Dexamethasone and Those in Osteoclast Formation in Co-Culture System

Osteoblast like stroma cell line, ST2, was cultured in the presence of active-form vitamin D₃ and dexamethasone for 7 days in the same manner as in Example 4-(3). OCIF-binding experiment was conducted using ¹²⁵I-labeled OCIF (monomer type) described in Example 4-(1). Nonspecific binding was measured by competing the ¹²⁵I-labeled OCIF with 400-fold higher concentration of unlabeled OCIF in binding to ST2 cell. As a result, the amount of specific binding of the ¹²⁵I-labeled OCIF was increased, due to active-form vitamin D₃ and dexamethasone, with increase of culturing days. That is, as shown in Figures. 4 and 5, the protein of the present invention was expressed on the cell surface of ST2 cell due to active-form vitamin D₃ with an increase of culturing days, and its expression reached maximum on the fourth day of culture. On the other hand, osteoclast like cell was formed after co-culture of mouse spleen cell and ST2 cell in the presence of active-form vitamin D₃. TRAP (a marker enzyme for osteoclast)-positive mononuclear osteoclast like cell was formed on the third or fourth day of culture, and, further, differentiated and matured TRAP-positive multinuclear cells were formed on the fifth or sixth day of culture. It was found that change with the passage of time in expression of the protein of the present invention and that in osteoclast formation were well corresponded each other.

(3) Effect of Inhibiting Osteoclast Formation When OCIF Was Treated Only during a Restricted Period of Co-Culture

To further clarify that the protein of the present invention was a factor involved in osteoclast formation, cells

cultured during various periods (two days each, except for the fifth day) were treated with 100 ng/ml of OCIF in the above 6-day coculture described in the Example 5-(2). As a result shown in Fig. 6, in case that OCIF was treated during 48th to 96th hr counted from the beginning of culture, when the protein of the present invention was expressed at highest level on ST2 cell, osteoclast formation was inhibited most effectively. That is, it was revealed that OCIF inhibited osteoclast formation by its binding to ST2 cell via the protein of the present invention.

From the above results, it became clear that the protein of the present invention was induced on the membrane of osteoblast like stroma cell with active-form vitamin D₃ and dexamethasone, and had the biological activity (effect) as a factor which supports or promotes differentiation and maturation of osteoclast.

Crosslinking Experiment of ¹²⁵I-labeled OCIF to the Protein of the Present Invention

To further identify the presence of the protein of the present invention, ¹²⁵I-labeled OCIF was allowed to crosslink with the protein of the present invention. As in Example 4-(3), mouse osteoblast like cell line, ST2, was cultured in the presence or absence of active-form vitamin D₃ and dexamethasone for 4 days. After the cells were washed with 1 ml of phosphate buffered saline solution, 200 µl of medium for binding experiment (α-MEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.2% NaN₃ and 100 µg/ml heparin) further containing 25 ng/ml of the above ¹²⁵I-labeled OCIF (monomer type) or 40 ng/ml of ¹²⁵I-labeled OCIF-CDD1 was added. ¹²⁵I-labeled OCIF-CDD1 which was obtained by expressing the protein described as SEQ ID No. 76 of Sequence Listing in WO96/26217 with animal cells and labeling in accordance with the above method. Furthermore, the medium for binding experiment containing 400-fold higher concentration of OCIF was added to other well and was subjected to an experiment for nonspecific binding. After the cells were cultured in a CO₂ incubator for 1 hour, they were washed three times with 1 ml of phosphate buffered saline solution containing 100 µg/ml of

heparin. Then, 500 μ l of phosphate buffered saline solution in which 100 μ g/ml of crosslinking agent DSS (Disuccinimidyl suberate, Pierce Co., Ltd.) was dissolved was added thereto, and allowed to react at 0°C for 10 minutes. After the cells in these wells were washed twice with 1 ml of phosphate buffered saline solution cooled to 0°C, 100 μ l of 20 mM Hepes buffer containing 1% Triton X-100, 2 mM PMSF (phenylmethylsulfonyl fluoride), 10 μ M pepstatin, 10 μ M leupeptin, 10 μ M antipain and 2 mM EDTA was added to each well, and left to stand at room temperature for 30 minute so as to lise the cells. After 15 μ l of these samples were treated with SDS under non-reducing conditions in accordance with a commonly used method, they were employed to electrophoresis using gel for SDS-polyacrylamide electrophoresis (with a gradient of 4 to 20% polyacrylamide, DAIICHI PURE CHEMICALS CO., LTD.). After electrophoresis, the gel was dried and exposed to BioMax MS film (Kodak Co., Ltd.) using BioMax MS amplifying screen (Kodak Co., Ltd.) at -80°C for 24 hours. The exposed films were developed in accordance with a commonly used method. When the 125 I-labeled OCIF (monomer type, 60 kDa) was used, a crosslinked protein having a molecular weight of about 90,000 to 110,000 was detected. On the other hand, when the 125 I-labeled OCIF-CDD1 (31 kDa) was used, a crosslinked protein of about 70 to 80 kDa (78 kDa on average) was detected as shown in Fig. 7.

[Example 7]

Scatchard Plot Analysis of the Protein of the Present Invention Expressed on ST Cell

Medium for binding experiment (α -MEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer and 0.2% NaN_3) further containing 1,000 pM of the above 125 I-labeled OCIF (monomer type) was prepared and diluted stepwise at a dilution rate of 1/2 with the medium for binding experiment. Furthermore, another medium for determining nonspecific binding was prepared by adding 400-fold higher concentration of unlabeled monomer type OCIF to the above medium. 200 μ l of these prepared solutions were added to wells of the above ST2 cells (about 10th passage) cultured for 4 days in the presence

of 10^{-8} M active-form vitamin D_3 (Calcitriol) and 10^{-7} M dexamethasone, and binding of the ^{125}I -labeled OCIF was tested in the same manner as in Example 4-(3). The obtained results were Scatchard-plotted in accordance with a common method, and dissociation constants of OCIF and the OCIF binding protein, and, the number of the OCIF binding protein (site) per one ST2 cell were determined. As a result, the dissociation constants of OCIF and the protein of the present invention were 280 pM, and the number of the OCIF binding protein (site) per one ST2 cell was about 33,000/cell. Furthermore, a cultured ST2 cell with a passage number of around 40's had higher ability to support osteoclast formation than that with a passage number of around 10's as shown in Example 5-(1), so that the number of sites of the protein of the present invention expressed on the ST2 cell with a passage number of around 40's was measured. As a result, the number of site was 58,000/cell which, was clearly greater than that on the ST2 cell with a passage number of around 10's. It was revealed that the amount of the expression of the protein of the present invention associated with the degree of the ability of ST2 cell to support osteoclast formation. This finding indicates that the protein of the present invention is a factor to support or promote differentiation and maturation of osteoclast.

[Example 8]

Cloning of OBMcDNA

(1) Extraction of RNA from Mouse ST2 Cell

Mouse osteoblast like stroma cell line, ST2, (RIKEN CELL BANK, RCB0224) was with α -MEM medium (GIBCO BRL CO., LTD.) containing 10% bovine fetal serum. After cultured cells become confluent in 225-cm² T flasks for adherent cell culture, ST2 cells were treated with trypsin, stripped from the T flask, washed, and transferred to five of 225-cm² T flasks. After adding 60 ml of α -MEM medium containing 10^{-8} M active-form vitamin D_3 (Calcitriol, Wako Pure Chemical Industries, Ltd.), 10^{-7} M dexamethasone and 10% bovine fetal serum thereto, the cells were cultured in a CO₂ incubator for 5 days. Total RNA was extracted from the cultured ST2 cells using ISOGEN (Wako Pure Chemical Industries, Ltd.). Poly A⁺ RNA was

prepared from about 600 µg of the total RNA using an Oligo(dT)-cellulose column (5'-3' Prime Co., Ltd.). About 8 µg of poly A⁺ RNA was obtained.

(2) Construction of Expression Library

5 Double strand cDNAs were synthesized from 2 µg of the poly A⁺ RNA obtained in Example 8-(1) with Great Lengths cDNA Synthesis kit (Clontech Co., Ltd.) in accordance with a manual thereof. More specifically, 2 µg of the poly A⁺ RNA and an Oligo(dT)₂₅(dN) primer were mixed together, distilled water was
10 added thereto so that the final volume was 6.25 µl, and the mixture was incubated at 70°C for 3 minutes, and then cooled in ice for 2 minutes. Then, 2.2 µl of distilled water, 2.5 µl of 5X First-strand buffer, 0.25 µl of 100 mM DTT (dithiothreitol), 0.5 µl of PRIME PNase Inhibitor (1 U/ml) (5'-
15 3' Prime Co., Ltd.), 0.5 µl of [α -³²P]dCTP (Amersham Co., Ltd., 3,000 Ci/mmol, 2 µCi/µl) which was diluted to be one fifth concentration, 0.65 µl of dNTP (20 mM each) and 1.25 µl (250 units) of MMLV (RNaseH⁻) reverse transcriptase were added thereto, respectively. Thus obtained solution was incubated at
20 42°C for 90 minutes. Then, 62.25 µl of distilled water, 20 µl of 5X second-strand buffer, 0.75 µl of dNTP (20 mM each) and 5 µl of Second-strand enzyme cocktail were added thereto, respectively. Thus obtained solution was incubated at 16°C for 2 hours. 7.5 units of T4 DNA polymerase was added thereto,
25 and further incubated at 16°C for another 30 minutes. Thereafter, 5 µl of 0.2 M EDTA was added to terminate the reaction, and after a phenol-chloroform treatment, ethanol precipitation was carried out. An EcoRI-SalI-NotI linker (Clontech Co., Ltd.) was added to an end of the double strand
30 cDNA and then phosphorylated at its end. Using a column for size fractionation, cDNAs of not smaller than 500 bp were separated, and ethanol-precipitated. The precipitated DNAs were reconstituted in water and inserted into pcDL-SR α 296 (Molecular and Cellular Biology, Vol. 8, pp. 466 to 472, 1988)
35 (TAKARA SHUZO CO., LTD.) previously cleaved with a restriction enzyme, EcoR1, and subsequently treated with CIAP (bovine

small intestine alkali phosphatase, TAKARA SHUZO CO., LTD.).

(3) Screening of Expression Library in Which the Binding to OCIF Was Used as Index

E. coli XL2 Blue MRF' (TOYOBO CO., LTD.) was
5 transformed with the DNA obtained in Example 8-(2), and
allowed to grow on a L Carbenicillin Agar Medium (1% trypton,
0.5% yeast extract, 1% NaCl, 60 µg/ml carbenicillin and 1.5%
agar) prepared in a 24-well plastic plate for cell culture so
that the cells was grown to about 100 colonies per well. The
10 transformants in each well were suspended in 3 ml of Terrific
Broth ampicillin medium (1.2% trypton, 2.4% yeast extract,
0.4% glycerol, 0.017 M KH_2PO_4 , 0.072 M K_2HPO_4 , 100 µg/ml
ampicillin), and cultured with shaking at 37°C overnight. The
E. coli was collected by centrifugation, and plasmid DNAs were
15 prepared therefrom with QIAwell kit (QIAGEN CO., LTD.). The
DNA content was determined by detecting absorbance at 260 nm,
and the DNAs were concentrated by ethanol precipitation and
dissolved in distilled water so that the concentration was 200
ng/µl. Thus, 500 DNA pools each derived from about 100
20 colonies were prepared and used for transfection of COS-7
cells (RIKEN CELL BANK, RCB0539). COS-7 cells were seeded in
a 24-well plate so as to achieve 8×10^4 cells/well and
cultured in a CO_2 incubator at 37°C overnight by use of a DMEM
medium containing 10% bovine fetal serum. On the following
25 day, the medium was removed, and the cells were then washed
with serum-free DMEM medium. In accordance with a protocol
attached to lipofectamine (GIBCO BRL CO., LTD.) which was a
reagent for transfection, the plasmid DNA previously diluted
with OPTI-MEM medium (GIBCO CO., LTD.) and lipofectamine were
30 mixed together, and after 15-minute incubation, the mixture
was added to the cells in each well. The amounts of DNA and
lipofectamine used were 1 µg and 4 µl, respectively. After 5-
hour incubation, the medium was removed, and 1 ml of DMEM
medium (GIBCO CO., LTD.) containing 10% bovine fetal serum was
35 added and cultured in a CO_2 incubator (5% CO_2) at 37°C for 2 to
3 days. The COS-7 cells obtained after transfection and
subsequent culture for 2 to 3 days were washed with serum-free
DMEM medium. Then, 200 µl of medium for binding experiment

(serum-free DMEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.1 mg/ml heparin and 0.02% NaN₃) further containing 20 ng/ml of ¹²⁵I-labeled OCIF was added thereto. Cells were cultured in a CO₂ incubator (5% CO₂) at 37°C for 1 hour and washed twice with 500 µl of phosphate buffered saline solution containing 0.1 mg/ml heparin. After washing, 500 µl of 0.1 N NaOH solution was added thereto, and then left to stand at room temperature for 10 minutes so as to lise the cells. The amount of ¹²⁵I in each well was measured with a gamma counter (Packard Co., Ltd.). After screening the 500 pools in total, a DNA pool containing a cDNA encoding a protein that could specifically bind OCIF was isolated. Furthermore, the DNA pools containing the cDNA of the present invention were subfractionated, and then amplyed to repeat the above transfection and screening. Thereafter, a cDNA encoding a protein which could bind OCIF was isolated. A plasmid containing the cDNA was referenced pOBM291. E. coli containing the plasmid was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology as pOBM291 with the deposition number of FERM BP-5953 on May 23, 1997. Methods for ¹²⁵I-labeling of OCIF and determining (the concentration of) ¹²⁵I-labeled OCIF by ELISA are as follows. OCIF was ¹²⁵I-labeled in accordance with Iodogen method. 20 µl of 2.5 mg/ml Iodogen-chloroform solution was transferred to a 1.5 ml Eppendorf tube, and chloroform was evaporated at 40°C so as to prepare an Iodogen-coated tube. After the tube was washed three times with 400 µl of 0.5 M sodium phosphate buffer (Na-Pi, pH: 7.0), 0.5 µl of 0.5 M Na-Pi with a pH of 7.0 was added thereto. Immediately after 1.3 µl (18.5 MBq) of Na-¹²⁵I solution (Amersham Co., Ltd., NEZ-033H20) was added thereto, 10 µl of 1 mg/ml OCIF solution (monomer type or dimmer type) was added. The resulting solution was agitated with a vortex mixer and then left to stand at room temperature for 30 seconds. This solution was transferred to a tube in which 10 mg/ml potassium iodide, 80 µl of 0.5 M Na-Pi solution (pH7.0) and 5 µl of phosphate buffered saline solution containing 5% bovine serum albumin (BSA-PBS) was previously

added and then agitated. This solution was applied to a spin column (1 ml, G-25 fine, Pharmacia Co., Ltd.) equilibrated with BSA-PBS and centrifuged at 2,000 rpm for 5 minutes.

After 400 µl of BSA-PBS was added to an eluate from the column

5 and mixed, 2 µl was subfractionated and its radioactivity was measured with a gamma counter. The radiochemical purity of thus prepared ^{125}I -labeled OCIF solution was determined by

measuring the radioactivity of a fraction precipitated with 10% TCA. Furthermore, the biological activity as OCIF of the

10 ^{125}I -labeled OCIF solution was determined in accordance with a method described in WO96/26217. Moreover, the concentration of ^{125}I -labeled OCIF was measured by ELISA in the following

manner. That is, 100 µl of 50 mM NaHCO_3 (pH: 9.6) in which 2 µg/ml of anti-OCIF rabbit polyclonal antibody described in

15 WO96/26217 was dissolved was added to each well of a 96-well immunoplate (Nunc Co., Ltd., MaxiSorpTM) and left to stand at

4°C overnight. After this solution was removed, 200 µl of a combined solution of of BLOCKACE (Snow Brand Milk Products

20 Co., Ltd.) and phosphate buffered saline solution (mixing ratio = 25:75: B-BPB) was added to each well and then left to

stand at room temperature for 2 hours. After this solution was removed, each well was washed three times with phosphate buffered saline solution (P-PBS) containing 0.01% Polysorbate

80. Thereafter, 100 µl of B-PBS containing a ^{125}I -labeled OCIF

25 or standard OCIF was added thereto and left to stand at room temperature for 2 hours. After this solution was removed,

each well was washed six times with 200 µl of P-PBS. Then, a peroxidase-labeled anti-OCIF rabbit polyclonal antibody was

diluted with B-PBS and 100 µl of the diluted solution was added

30 to each well, and then left to stand at room temperature for 2 hours. After this solution was removed, each well was washed

six times with 200 µl of P-PBS. Then, 100 µl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was

added to each well and then left to stand at room temperature

35 for 2 to 3 minutes. Thereafter, 100 µl of Stopping Reagent

(Scytek Co., Ltd.) was added thereto. The absorbance of each well at 450 nm was measured with a microplate reader. The

concentration of the ^{125}I -labeled OCIF was determined from a calibration curve made using standard OCIF.

(4) Determination of the Nucleotide Sequence of cDNA Which
Encodes the Full Length Amino Acid Sequence of OBM

5 The Nucleotide Sequence of OBMcdNA obtained in Example
8-(3) was determined with Taq Dye Deoxy Terminator Cycle
Sequencing kit (Perkin Elmer Co., Ltd.). That is, using
pOBM291 as a template, the Nucleotide Sequence of the inserted
10 fragment was directly determined. Furthermore, about 1.0 kb
and about 0.7 kb fragments obtained by cleaving pOBM291 with a
restriction enzyme, EcoRI, were inserted into EcoRI site of
plasmid pUC19 (TAKARA SHUZO CO., LTD.) and sequenced,
respectively. A primer SRR2 for sequencing the DNA fragment
inserted in pcDL-SR α 296, primers M13PrimerM3 and M13PrimerRV
15 (TAKARA SHUZO CO., LTD.) for sequencing the DNA fragment
inserted in the plasmid pUC19, and a synthetic primer OBM #8
designed based on the Nucleotide Sequence of OBMcdNA were
used. The sequences of these primers are shown as SEQ ID Nos.
3 to 6 of Sequence Listing.

20 Furthermore, the determined Nucleotide Sequence of
OBMcdNA is shown as SEQ ID No. 2, and the deduced an amino
acid sequence is shown as SEQ ID NO. 1.

[Example 9]

Expression of the Protein Encoded by the cDNA of the Present
25 Invention

30 COS-7 cells were transfected with plasmid pOBM291 with
lipofectamine in each well of a 6-well plate, and were
cultured in DMEM medium containing 10% bovine fetal serum for
2 days. The medium was replaced with cysteine/methionine-free
DMEM (DAINIPPON PHARMACEUTICAL CO., LTD.) in which 5% dialyzed
bovine fetal serum (800 μl /well) was added, and the cells were
cultured for another 15 minutes. Then, 14 μl of Express
Protein Labeling Mix (NEN CO., LTD., 10 mCi/ml) was added
thereto. After the cells were cultured for 4 hours, 200 μl of
35 DMEM medium containing 10% bovine fetal serum was added, and
the cells were cultured for 1 hour. After the cells were
washed twice with PBS, 0.5 ml of TSA buffer (10 mM Tris-HCl
(pH: 8.0) containing 0.14 M NaCl and 0.025% NaN_3) containing 1%

Triton X-100, 1% bovine hemoglobin, 10 µg/ml leupeptin, 0.2 TIU/ml aprotinin and 1 mM PMSF was added, and the cells were left to stand on ice for 1 hour. After the cells were crushed by pipetting, centrifugation was carried out at 3,000Xg at 4°C and for 10 minutes so as to obtain a supernatant. To 100 µl of this supernatant, 200 µl of dilution buffer (TSA buffer containing 0.1% Triton X-100, 0.1% bovine hemoglobin, 10 µg/ml leupeptin, 0.2 TIU/ml aprotinin and 1 mM PMSF) was added, and the resulting supernatant was shaken together with protein A Sepharose (50 µl) at 4°C for 1 hour, and then centrifuged at 4°C and 1,500 X g for 1 minute so as to collect a supernatant. Thereby, a fraction nonspecifically binding to the protein A Sepharose was removed. OCIF (1 µg) was added to this supernatant, and the obtained supernatant was shaken at 4°C for 1 hour so that OBM bound OCIF. Then, an anti-OCIF polyclonal antibody (50 µg) was added, and the solution was shaken at 4°C for 1 hour. Then, protein A Sepharose (10 µl) was further added, and the solution was further shaken at 4°C for another 1 hour. The solution was centrifuged at 1,500Xg at 4°C for 1 minute and the precipitated fraction was collected. The precipitate resulting from centrifugation at 1,500Xg at 4°C for 1 was washed twice with the dilution buffer, twice with the dilution buffer without bovine hemoglobin, once with TSA buffer, and once with 50 mM Tris-HCl (pH: 6.5). After washing, SDS buffer (0.125 M Tris-HCl, 4% dodecyl sodium sulfate, 20% glycerol, 0.002% bromophenol blue, pH: 6.8) containing 10% β mercaptoethanol was added to the precipitate. The precipitate was heated at 100°C for 5 minutes and subjected to SDS-PAGE (12.5% polyacrylamide gel, DAIICHI PURE CHEMICALS CO., LTD.). After the gel was fixed in accordance with a commonly used method, signals of isotope were amplified with Amplify (Amersham Co., Ltd.), and the fixed gel was exposed to Bio Max MR film (KODAK CO., LTD.) at -80°C. The results are shown in Fig. 8. The molecular weight of the protein encoded by the cDNA of the present invention was found to be about 40,000.

[Example 10]

Binding of the Protein Encoded by the cDNA of the Present
Invention to OCIF

COS cells was transfected with plasmid pOBM291 with lipofectamine in wells of a 24-well plate and cultured for 2 to 3 days. Then, the cells were washed with serum-free DMEM medium, and 200 μ l of medium for binding experiment (serum-free DMEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.1 mg/ml heparin and 0.2% NaN₃) containing 20 ng/ml of ¹²⁵I-labeled OCIF was added thereto. Furthermore, 200 μ l of the medium for binding experiment containing 8 μ g/ml of unlabeled OCIF in addition to 20 ng/ml of the ¹²⁵I-labeled OCIF was added to other wells. The cells were cultured in a CO₂ incubator (5% CO₂) at 37°C for 1 hour, and washed twice with 500 μ l of phosphate buffered saline solution containing 0.1 mg/ml heparin. After washing, 500 μ l of 0.1 N NaOH solution was added to each well, and then the well was left to stand at room temperature for 10 minutes so as to lise the cell. The amount of ¹²⁵I in each well was measured with a gamma counter. As a result, it was confirmed that the ¹²⁵I-labeled OCIF bound only to cells transfected with the plasmid pOBM291 as shown in Fig. 9. Further, it was also confirmed that the binding was significantly inhibited by addition of 400-fold higher concentration of (unlabeled) OCIF. From these results, it was revealed that OBM, a protein encoded by the cDNA of the plasmid pOBM291 specifically bound OCIF on the surface of COS-7 cell.

[Example 11]

Crosslinking Experiment of ¹²⁵I-labeled OCIF to the Protein
Encoded by the cDNA of the Present Invention

In order to analyze the characteristics of the protein encoded by the cDNA of the present invention, more specifically, ¹²⁵I-labeled monomer type OCIF was allowed to crosslink with the protein encoded by the cDNA of the present invention. COS-7 cells were transfected with plasmid pOBM291 in accordance with the method described in Example 8-(3), 200 μ l of medium for binding experiment containing the above ¹²⁵I-labeled OCIF (25 ng/ml) was added thereto. Furthermore, the medium for binding experiment containing 400-fold higher

concentration of unlabeled OCIF in addition to the ^{125}I -labeled OCIF was added to other wells. The cells were cultured in a CO_2 incubator (5% CO_2) at 37°C for 1 hour and washed twice with 500 μl of phosphate buffered saline solution containing 0.1 mg/ml of heparin. 500 μl of phosphate buffered saline solution containing 100 $\mu\text{g/ml}$ of crosslinking agent DSS (Disuccinimidyl suberate, Pierce Co., Ltd.) was added to these cells, and the cells were allowed to react at 0°C for 10 minutes. After the reaction, the cells in these wells were washed twice with 1 ml of phosphate buffered saline solution cooled to 0°C . Then, 100 μl of 20 mM Hepes buffer containing 1% Triton X-100 (Wako Pure Chemical Industries, Ltd.), 2 mM PMSF (phenylmethylsulfonyl fluoride, sigma Co., Ltd.), 10 μM pepstatin (Wako Pure Chemical Industries, Ltd.), 10 μM leupeptin (Wako Pure Chemical Industries, Ltd.), 10 μM antipain (Wako Pure Chemical Industries, Ltd.) and 2 mM EDTA (Wako Pure Chemical Industries, Ltd.) was added to these cells, and the wells were left to stand at room temperature for 30 minute so as to lise the cells. After 15 μl of these samples were treated with SDS under non-reducing conditions in accordance with a commonly used method, they were subjected to electrophoresis with gel for SDS-electrophoresis (gradient of 4 to 20% polyacrylamide, DAIICHI PURE CHEMICALS CO., LTD.). After electrophoresis, the gel was dried and exposed to BioMax MS film (Kodak Co., Ltd.) with BioMax MS amplifying screen (Kodak Co., Ltd.) at -80°C for 24 hours. The exposed films were developed in accordance with a commonly used method. As results of crosslinking of the ^{125}I -labeled monomer type OCIF with the protein encoded by the cDNA of the present invention, a band having a molecular weight of about 90,000 to 110,000 was detected as shown in Fig. 10.

[Example 12]

Northern Blot Analysis

ST2 cells were cultured to become confluent in a 25- cm^2 T flask for culturing adherent cells, and treated with trypsin. After stripped from the T flask, the cells were washed and seeded in a 225- cm^2 T flask. 60 ml of α -MEM medium

containing 10^{-8} M active-form vitamin D₃, 10^{-7} M dexamethasone and 10% bovine fetal serum was added thereto, the cells were cultured in a CO₂ incubator for 4 days. Total RNA was extracted from the cultured ST2 cells with ISOGEN (Wako Pure Chemical Industries, Ltd.). Furthermore, total RNA was extracted from ST2 cells cultured in the absence of the active-form vitamin D₃ and dexamethasone in accordance with the above method. To 20 µg (4.5 µl) of each total RNA, 2.0 µl of 5X gel electrophoresis buffer (0.2 M morpholinopropanesulfonic acid, pH: 7.0, 50 mM sodium acetate, 5 mM EDTA), 3.5 µl of formaldehyde and 10.0 µl of formamide were added. The total RNAs were incubated at 55°C for 15 minutes and subjected to electrophoresis. Gel for electrophoresis consisted of 1.0% agarose, 2.2 M ionized formaldehyde, 40 mM morpholinopropanesulfonic acid (pH: 7.0), 10 mM sodium acetate and 1 mM EDTA. Moreover, the electrophoresis was performed in buffer comprising 40 mM morpholinopropanesulfonic acid (pH: 7.0), 10 mM sodium acetate and 1 mM EDTA. After the electrophoresis, the RNAs were transferred to nylon membranes. About 1.0 kb DFA fragments were obtained by cleaving pOBM291 with a restriction enzyme, EcoRI, and labeled with α -³²P-dCTP (Amersham Co., Ltd.) using MEGAPRIME DNA Labeling Kit (Amersham Co., Ltd.), and thus used as probes for hybridization. As a result, it was revealed that gene expression of the protein (OBM) encoded by the cDNA of the present invention was strongly induced in the ST2 cell cultured in the presence of active-form vitamin D₃ and dexamethasone.

[Example 13]

Ability of the Protein Encoded by the cDNA of the Present Invention to Support Osteoclast formation

In accordance with the method described in Example 8-(3), COS cells were transfected with pOBM219. After 3-day incubation, the cells were treated with trypsin and then centrifuged-washed once with phosphate buffered saline solution. Then, the cells were fixed at room temperature for 5 minutes in suspension of PBS containing 1% paraformaldehyde, and then centrifuged-washed six times with PBS. Mouse spleen

cells and ST2 cells were prepared with α -MEM medium containing 10^{-8} M active-form vitamin D₃, 10^{-7} M dexamethasone and 10% bovine fetal serum so that the cell concentration become 1×10^6 cells/ml or 4×10^4 cells/ml and then added to a 24-well plate in a volume of 700 μ l and 350 μ l, respectively. Furthermore, TC insert (Nunc Co., Ltd.) was set in each well. The fixed COS cells (350 μ l) diluted stepwise with the above medium and OCIF (50 μ l) were added to TC inserts and cultured at 37°C for 6 days. As a result, it was revealed that an activity of OCIF to inhibit osteoclast formation was suppressed with the protein encoded by the cDNA of the present invention.

[Example 14]

Expression of Secretory-type OBM

(1) Construction of Plasmid for Expressing Secretory-Type OBM

Expression

A PCR reaction was carried using OBM HF (SEQ ID No. 7 of Sequence Listing)/OBM XR (SEQ ID No. 8 of Sequence Listing) and pOBM291 as primers and a template, respectively. After the reaction product was purified through agarose gel electrophoresis, it was cleaved with the restriction enzymes, HindIII and EcoRI, and then purified through agarose gel electrophoresis again. The purified fragment (0.6 kb), HindIII/EcoRV fragment (5.2 kb) of pSec TagA (Invitrogen Co., Ltd.) and EcoRI/PmaCI fragment (0.32 kb) of OBM cDNA was subjected to ligation using Ligation Kit Ver. 2 (TAKARA SHUZO CO., LTD.), and subsequently E. coli DH5 α was transformed by the ligation product. Plasmid was purified from the obtained ampicillin-resistant strains by alkaline-SDS method and then cleaved with restriction enzymes so as to select a plasmid wherein 0.6 kb and 0.32 kb of fragments were inserted into pSec TagA. The selected plasmid was subjected to sequencing with Dye Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.), thereby it was confirmed that the plasmid had the sequence encoding secretory-type OBM (Nucleotide Sequence: 338th to 1,355th of SEQ ID NO. 2 of Sequence Listing, amino acid sequence: 72th to 316th of SEQ ID NO. 1 of Sequence Listing). After the plasmid was cleaved with restriction enzymes, NheI and XhoI, a fragment (1.0 kb) corresponding to

secretory-type OBM cDNA was collected by agarose gel electrophoresis. This fragment was inserted into NheI/XhoI fragment (10.4 kb) of an expression vector, pCEP4 (Invitrogen Co., Ltd.), by using the ligation kit, and E. coli DH5 α was transformed by the ligation product. Plasmid was purified from the obtained ampicillin-resistant strains by alkaline-SDS method, and cleaved with the restriction enzymes and then analyzed so as to select E. coli strain which had a plasmid for expressing secretory-type OBM (pCEP sOBM) with the desired structure. The E. coli strain having the pCEP sOBM was cultured, and the pCEP sOBM was purified therefrom with QIA Filter Plasmid Midi Kit (QIAGEN CO., LTD.).

(2) Expression of Secretory-Type OBM

293-EBNA cells were suspended in IMDM containing 10% FCS (IMDM-10%FCS), and seeded in a collagen-coated 24 well plate (SUMITOMO BAKELITE CO., LTD.) so that the cell concentration was 2×10^5 cells/2 ml/well, and cultured overnight. The cells were transduced by 1 μ g of pCEP sOBM or pCEP4 using 4 μ l of lipofectamine (GIBCO CO., LTD.), and then cultured in 0.5 ml of serum-free IMDM or IMDM-10%FCS for another 2 days. Thereafter, the conditioned medium was collected. Expression of secretory-type OBM in the conditioned medium was confirmed in the following manner. After sodium hydrogencarbonate was added to the conditioned medium so that the final concentration was 0.1 M, the culture solution was added to a 96-well plate, and left to stand at 4°C overnight, and then OBM in the conditioned medium was immobilized in the 96-well plate. This plate was left to stand for blocking at room temperature for 2 hours by use of BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution diluted with PBS to be one fourth concentration (B-PBS). Then, 100 μ l of 3-100 ng/ml OCIF diluted with B-PBS was added to each well, and the wells were left to stand at 37°C for 2 hours. After washing the plate with PBS (PBS-T) containing 0.05% Tween 20, 100 μ l of peroxidase-labeled anti-OCIF rabbit polyclonal antibody, which was described in WO96/26217, diluted with B-PBS was added to each well, and the cells were left to stand at 37°C for 2 hours. After washing each well with PBS-T six

times, 100 µl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added thereto and then left to stand at room temperature for about 10 minutes.

Thereafter, 100 µl of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at 450 nm was measured with a microplate reader. The results are shown in Fig. 12. In the plate in which substances included in the conditioned medium of the cells transduced by pCEP sOBM was immobilized, absorption at 450 nm increased in the OCIF concentraion-dependent manner. On the other hand, in the plate in which substances included in the conditioned medium of the cells transduced by pCEP4 vector was immobilized, no increase in absorption at 450 nm was observed. Furthermore, Fig. 13 shows the results of experiments when the amount of the conditioned medium applied to the immobilization was varied within a range of 5 to 90% and a constant concentration of OCIF (50 ng/ml) was futher added. In the plate in which substances included in the conditioned medium of the cells transduced by pCEP sOBM was immobilized, absorption at 450 nm increased corresponding to an increase in the amount of the conditioned medium. On the other hand, in the plate in which substances included in the conditioned medium of the cells transduced by pCEP4 vector was immobilized, no increase in absorption was observed. From these results, secretory-type OBM was onfirmed to be produced in the conditioned medium of the cells transduced by pCEP sOBM.

[Example 15]

Expression of Thioredoxin-OBM Fusion Protein (Trx-OBM)

(1) Construction of a Vector for Expressing Thioredoxin-OBM Fusion Protein (Trx-OBM)

10 µl of 10X ExTaq buffer (TAKARA SHUZO CO., LTD.), 8 µl of 10 mM dNTP (TAKARA SHUZO CO., LTD.), 77.5 µl of sterilized distilled water, 2 µl of pOBM291 solution (10 ng/µl), 1 µl of primer OBM3 (100 pmol/µl, SEQ ID NO. 9 of Sequence Listing), 1 µl of primer OBMSalR2 (100 pmol/µl, SEQ ID NO. 10 of Sequence Listing) and 0.5 µl of ExTaq (5µ/µl) (TAKARA SHUZO CO., LTD.) were mixed together, and then PCR reaction was conducted in a microtube for centrifugation. After the

reaction was carried out at 95°C for 5 minutes, 50°C for 1 second, 55°C for 1 minute, 74°C for 1 second and 72°C for 5 minutes, the cycle reaction consisting of at 96°C for 1 minute, 50°C for 1 second, 55°C for 1 minute, 74°C for 1 second and 72°C for 3 minutes was repeated 25 times. Through 1% agarose gel electrophoresis, about 750 bp of DNA fragment was purified from whole the reaction solution with QIAEX II Gel Extraction Kit (QIAGEN CO., LTD.). All amount of the purified DNA fragment was cleaved with restriction enzymes SalI and EcoRI (TAKARA SHUZO CO., LTD.), and about 160-bp DNA fragment(fragment 1) was purified through 1.5% agarose gel electrophoresis and dissolved in 20 µl of sterilized distilled water. Similarly, 4 µg of pOBM291 and 2 µg of pTrxFus (InVitrogen Co., Ltd.) were cleaved with restriction enzymes BamHI/EcoRI and BamHI/SalI (TAKARA SHUZO CO.LTD.), respectively, and about 580-bp DNA fragment (fragment 2) and about 3.6-kb DNA fragment (fragment 3) were purified therefrom and dissolved in 20 µl of sterilized distilled water, respectively. QIAEX II Gel Extraction Kit was used for for purifying the fragments. Fragments 1, 2 and 3 were ligated by incubating them using DNA Ligation Kit Ver. 2 (TAKARA SHUZO CO., LTD.) at 16°C for 2.5 hours. Then, E. coli GI724 strain (InVitrogen Co., Ltd.) was transformed with the ligation product in accordance with the method described in an instruction manual attached to ThioFusion Expression System (InVitrogen Co., Ltd.). Among the resulting ampicillin-resistant transformants, one having a plasmid, in which an OBMcdNA fragment (Nucleotide Sequence: 350th to 1,111th of SEQ ID NO. 2 of Sequence Listing, amino acid sequence: 76th to 316th of SEQ ID NO. 1 of Sequence Listing) was linked to thioredoxin gene in the same reading frame, was selected after analysis of DNA fragment map obtained by restriction enzyme cleavage and DNA sequencing. The obtained strain was referenced GI724/pTrxOBM25.

(2) Expression of OBM in E. coli

GI724/pTrxOBM25 strain and GI724 strain having pTrxFus (GI724/pTrxFus) were cultured in 2 ml of RMG-Amp medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 1.2% casamino acid

(Difco Co., Ltd.), 1% glycerol, 1 mM MgCl₂, 100 µg/ml ampicillin (sigma Co., Ltd.), pH: 7.4) with shaking at 30°C for 6 hours. 0.5 ml of the cell suspension was added to 50 ml of Induction medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 0.2% casamino acid, 0.5% glucose, 1 mM MgCl₂, 100 µg/ml ampicillin, pH: 7.4) and cultured with shaking at 30°C. L-tryptophan was added so that the final concentration was 0.1 mg/ml when the value at OD_{600 nm} became about 0.5, and the cells were further cultured at 30°C for 6 hours. The cell suspension was centrifuged at 3,000Xg and the collected cells were then suspended in 12.5 ml of PBS (10 mM phosphoric acid buffer, 0.15 M NaCl, pH: 7.4). The suspension was subjected to ultrasonication using a ultrasonicator (Ultrasonics Co., Ltd.) so that the cells were crushed, and then centrifuged at 7,000Xg for 30 minutes. The recovered supernatant was used as soluble protein fraction. 10 µl of the soluble protein fraction solution was subjected to SDS polyacrylamide (10%) electrophoresis under reducing conditions. As a result, a band having a molecular weight of about 40kDa was observed in the soluble protein fraction solution of GI724/pTrxOBM25, while not observed in soluble protein fraction solution of GI724/pTrxFus (Fig. 14). Thus, it was confirmed that the thioredoxin-OBM fusion protein (Trx-OBM) was expressed in E. coli.

(3) Binding Ability of Trx-OBM to OCIF

In the following experiment, it was confirmed that the expressed Trx-OBM bound to OCIF. Anti-thioredoxin antibody (InVitrogen Co., Ltd.) was diluted with 10 mM sodium hydrogencarbonate solution so that the concentration was 1/5,000, and 100 µl thereof was added to each well of a 96-well immunoplate (Nunc Co., Ltd.) and then left to stand at 4°C overnight. After the solution in each cell was discarded, 200 µl of 1/2 concentration of BLOCKACE (Snow Brand Milk Products Co., Ltd.) diluted with PBS (BA-PBS) was added to each well and then left to stand at room temperature for 1 hour. After the solution was discarded, 100 µl of the soluble protein fraction solution derived from GI724/pTrxOBM25 which was diluted stepwise with BA-PBS and 100 µl of that derived from

GI724/pTrxFus which was diluted stepwise with BA-BPB were added to wells and left to stand at room temperature for 2 hours, respectively. After washing each well three times with PBS-T, 100 μ l of OCIF (100 ng/ml) diluted with BA-PBS was added to each well and left to stand at room temperature for 2 hours. After washing each well three times with PBS-T, 100 μ l of peroxidase-labeled anti-OCIF rabbit polyclonal antibody described in WO96/26217, which was diluted with BA-PBS so that the concentration was 1/2,000, was added to each well and left to stand at room temperature for 2 hours. After washing each well six times with PBS-T, 100 μ l of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added thereto and then left to stand at room temperature for about 10 minutes. Thereafter, 100 μ l of Stopping Reagent (Scytek Co., Ltd.) was added thereto. Absorbance of each well at 450 nm was measured with a microplate reader. The results are shown in Fig. 15. The absorbance when added soluble protein fraction solution derived from GI724/pTrxFus was increased in concentration (of the added solution)-dependent manner, while no difference in absorbance was observed between when added soluble protein fraction solution derived from GI724/pTrxFus and when not added said soluble protein fraction solution. Furthermore, Fig. 16 shows the results of experiments when the dilution rate of soluble fraction solution was kept constant (1%) and OCIF diluted stepwise with BA-PBS (0-100 ng/ml) was further added. Absorbance was kept low regardless of the concentration of OCIF when added soluble protein fraction solution derived from GI724/pTrxFus. However, absorbance was increased in OCIF concentration-dependent manner when added soluble protein fraction solution derived from GI724/pTrxOBM25. Thus, it was confirmed that Trx-OBM produced in GI724/pTrxOBM25 had an ability to bind OCIF.

(4) Large Scale Culture of E. coli Producing Trx-OBM

GI724/pTrxOBM25 was spread on an RMG-Amp agar medium (0.6% Na_2HPO_4 , 0.3% KH_2PO_4 , 0.05% NaCl , 0.1% NH_4Cl , 2% casamino acid, 1% glycerol, 1 mM MgCl_2 , 100 $\mu\text{g/ml}$ ampicillin, 1.5% agar, pH: 7.4) with a platinum loop and cultured at 30°C overnight. The cells were suspended in 10 ml of Induction medium. 5 ml

of the suspension was added to two of 2L conical flask containing 500 ml of Induction medium and cultured with shaking at 30°C. L-tryptophan was added so that the final concentration was 0.1 mg/ml when OD_{600 nm} value became about 0.5, and then further cultured with shaking at 30°C for 6 hours. The cell suspension was centrifuged at 3,000Xg for 20 minutes and the cells were collected, and then suspended in 160 ml of PBS. The suspension was subjected to ustrasoniaction using an ultrasonicator (Ultrasonics Co., Ltd.) for crushing cells, and then centrifuged at 7,000Xg for 30 minutes. Thereafter, the supernatant was recovered as soluble protein fraction.

(5) Preparation of OCIF-immobilized Affinity Column

2 g of TSKgel AF-Tresyl TOYOPAL 650 (TOSO CO., LTD.) and 40 ml of 1.0 M potassium phosphate buffer (pH: 7.5) containing 35.0 mg of recombinant OCIF prepared by a method described in WO96/26217 were mixed together and gently shaken at 4°C overnight so as to cause a coupling reaction. After the supernatant was removed by centrifugation, 40 ml of 0.1 M Tris-hydrochloric acid buffer (pH: 7.5) was added to the precipitated carrier, and the mixture was gently shaken at room temperature for 1 hour, in order to inactivate an excess amount of active groups thereon. After washing the column with both 0.1 M glycine-hydrochloric acid buffer (pH: 3.3) containing 0.01% Polysorbate 80/0.2 M NaCl and 0.1 M sodium citrate buffer (pH: 2.0) containing 0.01% Polysorbate 80/0.2 M NaCl, the column was washed twice with 10 mM sodium phosphate buffer (pH: 7.4) containing 0.01% Polysorbate 80 and equilibrated therewith.

(6) Purification of Trx-OBM Using OCIF-immobilized Affinity Column

Purification of Trx-OBM was carried out at 4°C unless otherwise stated. The above OCIF-immobilized affinity carrier (10 ml) and the above soluble protein fraction solution (120 ml) described in Example 15-(4) were mixed together, the mixture was gently shaken in four of 50 ml cenctirugation tube with a rotor at 4°C overnight. The carrier in the mixture was embeded in Econo Column (Biorad Co., Ltd., internal diameter: 1.5 cm, length: 15 cm). The column was washed with 300 ml of PBS containing 0.01% Polysorbate 80, 100 ml of 10 mM sodium

phosphate buffer (pH: 7.0) containing 0.01% Polysorbate 80 and 2 M NaCl, and, 100 ml of 0.1 M glycine-hydrochloric acid buffer (pH: 3.3) containing 0.01% Polysorbate 80 and 0.2 M NaCl. Then, proteins was eluted from the column with 0.1 M sodium citrate buffer (pH: 2.0) containing 0.01% Polysorbate 80 and 0.2 M NaCl. 5 ml of eluates were fractionated. Immediately, 10% volume of 2M Tris solution (pH: 8.0) was added for neutralization. The presence or absence of Trx-OBM in the each fraction of the eluate was examined in accordance with the above method) described in Example 15-(3) (ability to bind OCIF. Fractions containing Trx-OBM were collected and further purified.

(7) Purification of Trx-OBM by Gel Filtration

Using Centriplus 10 and Centricon 10 (amicon Co., Ltd.), about 25 ml of the above Trx-OBM fraction of Example 15-(6) was concentrated by centrifugation to a final volume of about 0.5 ml. This sample was subjected to Superose 12 HR 10/30 column (1.0 X 30 cm, Pharmacia Co., Ltd.) previously equilibrated with PBS containing 0.01% Polysorbate 80. The column was developed using PBS containing 0.01% Polysorbate 80 as a mobile phase at a flow rate of 0.25 ml/min and 0.25 ml of eluates from the column were collected. Trx-OBM in the fractions was detected by the method described in Example 15-(3), and, SDS-polyacrylamide electrophoresis (gradient gel of 10 to 15% polyacrylamide, Pharmacia Co., Ltd.) using Phast System (Pharmacia Co., Ltd.) and silver staining. Fractions (Fr. 20 to 23) containing purified Trx-OBM were collected and subjected to measurement of the protein concentration of Trx-OBM. The measurement was carried out with DC-protein assay kit (Biorad Co., Ltd.), using bovine serum albumin as standard.

[Example 16]

Osteoclastogenesis Promoting Activity of OBM

COS-7 cells were transfected with pOBM291 and pcDL-SR α 296 using lipofectamine (GIBCO CO., LTD.), respectively. After the cells were cultured in DMEM containing 10% FCS for 1 day, they were treated with trypsin, and seeded in a 24 well plate, in which a cover glass (15 mm round, MATSUNAMI CO., LTD.) was seated, so that the concentration became 5×10^4

cells/well. The cells were then cultured for another two days. After washing the culture plate once with PBS, PBS containing 1% paraformaldehyde was added thereto, and the cells were incubated at room temperature for 8 minutes and fixed. After washing the plate in which the cells were fixed six times with PBS, 700 μ l of 1×10^6 cells/ml suspension of mouse spleen cell in α -MEM containing 10^{-8} M active-form vitamin D_3 , 10^{-7} M dexamethasone and 10% bovine fetal serum was added to each cell. Millicell PCF (Millipore Co., Ltd.) was set on each well, and 700 μ l of 4×10^4 cells/ml suspension of ST2 cell in the above medium was added to the Millicell PCF and cultured at 37°C for 6 days. After the culture, the Millicell PCF was removed, and the plate was washed once with PBS. The cells were fixed with acetone-ethanol solution (50:50) for 1 minute, and then only the cells showing tartaric acid resistant acid phosphatase activity (TRAP activity), a specific marker for osteoclast, were stained with LEUKOCYTE ACID PHOSPHATASE kit (Sigma Co., Ltd.). As a result of observation using a microscope, 45 ± 18 (average \pm standard deviation, $n = 3$) TRAP positive cells were observed in the wells in which pOBM291-transfected COS-7 cells were fixed, while no cells showing TRAP activity were detected in the wells in which pcDL-SR α 296-transfected COS-7 cells fixed. Furthermore, it was also confirmed that calcitonin bound said TRAP positive cells. Thereby, it was revealed that OBM had an activity to promote osteoclast formation.

[Example 17]

Osteoclastogenesis Promoting Activities of Trx-OBM and Secretory-type OBM

Mouse spleen cells were suspended in α -MEM containing 10^{-8} M active-form vitamin D_3 , 10^{-7} M dexamethasone and 10% bovine fetal serum in a concentration of 2×10^6 cells/ml, and 350 μ l of this suspension was added to each well of a 24-well plate. 350 μ l of the solution (40 ng/ml) obtained by diluting the purified Trx-OBM with the above medium, or, 350 μ l of solution obtained by diluting the conditioned medium of 293-EBNA cells (which were transduced by pCEP sOBM or pCEP4

cultured in IMDM-10%FCS with the above medium) so that the concentration was 1/10, or, 350 μ l of the above medium alone was added to each well. Then, Millicell PCF (Millipore Co., Ltd.) was set on each well, and 600 μ l of 4×10^4 cells/ml suspension of ST2 cell in the above medium were added to the Millicell PCF. After the cells were cultured for 6 days, the Millicell PCF was removed, and the plate was washed once with PBS. When the cells were fixed with acetone-ethanol solution (50:50) for 1 minute, only the cells showing tartaric acid resistant acid phosphatase activity (TRAP activity) were stained with LEUKOCYTE ACID PHOSPHATASE kit (sigma Co., Ltd.). As a result of observation using a microscope, 106 ± 21 (average \pm standard deviation, $n = 3$) TRAP positive cells were observed in the wells when added Trx-OBM was added, while no cells showing TRAP activity were detected in the wells when not added. Similarly, 120 ± 31 (average \pm standard deviation, $n = 3$) TRAP positive cells were observed in the wells when added the conditioned medium of 293-EBNA cells transduced by pCEP-sOBM, while no cells showing TRAP activity were detected in the wells when not added. Furthermore, it was confirmed that calcitonin bound to these TRAP positive cells. Thereby, it was revealed that Trx-OBM and secretory-type OBM had an activity to promote osteoclast formation.

[Example 18]

Identity of the Protein OBM Expressed by the cDNA of the Present Invention and Natural-Type OCIF Binding Protein of the Present Invention

(1) Preparation of Anti-OBM Rabbit Polyclonal Antibody

Three male Japanese white rabbits (weight: 2.5 to 3.0 kg, purchased from KITAYAMA LABETH CO., LTD.) were subjected to hypodermic immunization with 1 ml of emulsion prepared by mixing 200 μ g/ml of purified OBM (thioredoxin-OBM fusion protein), which was obtained in accordance with the methods described in Examples 14-(6) and 14-(7), with 200 μ g/ml of Freund's complete adjuvant (DIFCO CO., LTD.). The immunization was carried out 6 times in total with one-week interval each, and all the blood was collected from the rabbits on the 10th day counted from the last immunization. An

antibody was purified from the fractionated serum in the following manner. That is, the antiserum diluted with PBS to be 1/2 concentraion, and ammonium sulfate was added thereto so that the final concentration was 40% (w/v%). Then, the antiserum was left to stand at 4°C for 1 hour and centrifuged at 8,000Xg for 20 minutes. Thereafter, the precipitate was collected and dissolved in a small aliquot of PBS, and then dialyzed against PBS at 4°C. The resulting solution was charged onto a Protein G-Sepharose column (Pharmacia Co., Ltd.). After washing the column with PBS, immunoglobulin G adsorbed was eluted with 0.1 M glycine-hydrochloric acid buffer (pH: 3.0), and pH thereof was immediately adjusted to be neutral with 1.5 M Tris-hydrochloric acid buffer (pH: 8.7). After the eluted protein fraction was dialyzed against PBS, absorbance at 280 nm was measured and its concentration was determine ($E^{1\%}_{1\text{cm}}$ 13.5). Horseradish peroxidase-labeled anti-OBM antibody was prepared with Maleimide Activated Peroxidase Kit (Pierce Co., Ltd.). That is, 80 µg of N-succinimide-S-acetylthioacetic acid was added to 1 mg of the purified antibody and allowed to react at room temperature for 30 minutes. 5 mg of hydroxylamine was added thereto for deacetylation, and then the modified antibody was fractionated by using a polyacrylamide desalting column. The protein fraction was mixed with 1 mg of maleimide activated peroxidase and allowed to react at room temperature for 1 hour, and then the enzyme-labeled antibody was obtained.

(2) Inhibition of Specific Binding of the Protein Expressed by the cDNA of the Present Invention (OBM) or Natural-Type Protein of the Present Invention to OCIF by Anti-OBM Rabbit Polyclonal Antibody

2 µg/ml of purified OBM (thioredoxin-OBM fusion protein) obtained in accordance with the methods described in Examples 15-(6) and 15-(7), and, 2 µg/ml of natural-type purified OCIF binding protein of Example 2-(4) were dissolved in 0.1 M sodium hydrogencarbonate, respectively, and 100 µl of each solution was added to each well of a 96-well immunoplate (Nunc Co., Ltd.) and then left to stand at 4°C overnight. 200 µl of 50% BLOCKACE was added to each well and left to stand at

room temperature for 1 hour. After washing wells three times with PBS containing 0.1% Polysorbate 20 (P20-PBS), 200 µg/ml of anti-OBM rabbit antibody was dissolved in 25% BLOCKACE diluted with P20-PBS, and 100 µl of the antibody solution or 25% BLOCKACE without antibody was added to each well and incubated at 37°C for 1 hour. After washing wells three times with P20-PBS, 100 µl of medium for binding experiment (P20-PBS containing 0.2% bovine serum albumin, 20 mM Hepes and 0.1 mg/ml Heparin) containing 20 ng/ml of the ¹²⁵I-labeled OCIF described in Example 8-(3) was added thereto. Furthermore, 100 µl of medium for binding experiment containing 8 µg/ml of unlabeled OCIF in addition to 20 ng/ml of the ¹²⁵I-labeled OCIF was added to other wells. After incubating the immunoplate at 37°C for 1 hour, each well was washed six times with P20-PBS. The amount of ¹²⁵I in each well was measured with a gamma counter. The results are shown in Fig. 17. As shown in Fig. 17, neither OBM obtained by expressing the cDNA of the present invention and subsequently purifying, nor, the natural-type protein of the present invention which specifically binds OCIF bound the ¹²⁵I-labeled OCIF when treated with the anti-OBM polyclonal rabbit antibody. On the other hand, it was confirmed that both proteins bound to the ¹²⁵I-labeled OCIF when not treated with said antibody. Furthermore, it was also revealed that bindings of both proteins to OCIF were specific binding since the bindings were significantly inhibited by addition of 400-fold higher concentration of unlabeled OCIF (8 µg/ml). From the above results, it was revealed that the anti-OBM rabbit polyclonal antibody recognized both OBM which was a protein expressed by the cDNA of the present invention and the natural-type OCIF binding protein of the present invention, and inhibited specific bindings of both proteins to OCIF.

[Example 19]

Cloning of Human OBMcDNA

(1) Preparation of Mouse OBM Primer

For screening of human OBMcDNA, a mouse OBM primer prepared in accordance with the method of the above Example, OBM #3 and OBM #8 were used. Sequences thereof are shown in

SEQ ID NO 9 and SEQ ID NO 6 of the Sequence Listing.

(2) Acquisition of Human OBMcdNA Fragments by PCR

A human OBMcdNA fragments was obtained by a PCR method using Human Lymph Node Marathon ready cDNA (CLONTECH CO., LTD.) which was a human lymph node derived cDNA library as a mold and usig the mouse OBMcdNA primer prepared in the above (1).

The following are the conditions used for PCR.

	10 X EX Taq buffer (TAKARA SHUZO CO., LTD.	2 µl
10	2.5 mM dNTP	1.6 µl
	cDNA solution	1 µl
	EX Taq (TAKARA SHUZO CO., LTD.)	0.2 µl
	Distilled Water	14.8 µl
	40 µM Primer OBM #3	0.2 µl
15	40 µM Primer OBM #8	0.2 µl

After the above solutions were mixed together in a microfusee tube, PCR was conducted under the following conditions. A pretreatment was carried out at 95°C for 2 minutes then, the cysle reaction consisting of at 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 2.5 minutes, was repeated 40 times, the solution was incubated at 72°C for 5 minutes. When a subfraction of the reaction product to agarose-electrophoresis , about 690 bp of DNA fragments amplified with the above mouse OBMcdNA primers were detected.

(3) Purification of Human OBMcdNA Amplified by PCR and Determination of Nucleotide Sequence

The human OBMcdNA fragments obtained in Example 19-(2) were separated by agarose-electrophoresis and then purified by use of a QIAEX gel extraction kit (QIAGEN CO., LTD.). By use of the purified human OBMcdNA fragments as templates, PCR was conducted again by use of the above mouse OBMcdNA primer so as to prepare a large amount of human OBMcdNA fragments which were then purified by use of the QIAEX gel extraction kit. The nucleotide sequence of the purified human OBMcdNA fragment was determined by use of a Taq Dye Deoxy Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.) using OBM #3 and OBM #8 (SEQ ID NO:9 and 6 in the SEQUENCE LISTINGlisting) as

primers. As a result of comparing the nucliotide sequence of the human OBMcdNA fragment with the corresponding part of mouse OBMcdNA, they shared a homology of 80.7%.

(4) Screening of Full Length Human OBMcdNA by Hybridization

5 Method Using Human OBMcdNA Fragments With length of About 690 bp as Probes

The human OBMcdNA fragments with length of about 690 bp purified in Example 19-(3) were labeled with [$\alpha^{32}\text{P}$] dCTP by use of a MEGA PRIME DNA labeling kit (Amersham Co., Ltd.), and
10 full length human OBMcdNA was screened. As an object to be screened, a Human Lymph Node 5'-STRETCH PLUS cDNA library (CLONTECH CO., LTD., USA) was used. In accordance with a protocol issued by the company, after Escherichia coli C600 Hfl was infected with recombinant phage at 37°C for 15
15 minutes, the Escherichia coli was added to an LB agar medium (1% trypton, 0.5% yeast extract, 1% NaCl, 0.7% agar) heated at 45°C and poured onto an LB agar medium plate containing 1.5% agar. After overnight culturing at 37°C, HIBIND N (Amersham Co., Ltd.) was brought into intimate contact with the plate
20 having plaques formed thereon for about 3 minutes. Then, this filter was subjected to an alkaline denaturation treatment in accordance with a commonly used method, and neutralized. and immersed in a 2 X SSC solution, and a DNA was fixed on the filter by UV CROSSLINK (STRATAGENE CO., LTD.). The obtained
25 filter was immersed in a Rapid-hyb buffer (Amersham Co., Ltd.) and pretreated at 65°C for 15 minutes. Thereafter, the filter was transferred into the above buffer containing the above heat denatured human OBMcdNA fragments (about 690 bp, 5×10^5 cpm/ml) and allowed to hybridize at 65°C overnight. After the
30 reaction, the filter was washed with 0.1%-SDS-containing 2 X SSC once, with 1 X SSC once and with 0.1 X SSC once in turn at 65°C for 15 minutes. The obtained positive clones were further screened twice so to be purified. A clone having about 2.2 kb of insert was selected out of these and used in
35 the following experiment. The purified phage was named λhOBM . From the purified λhOBM , about 10 μg of DNA was obtained in accordance with a protocol of a QIAGEN Lambda kit (QIAGEN CO., LTD.). After this DNA was cleaved with a restriction enzyme

SalI, about 2.2 kb of hOBM insert cDNA was separated by agarose electrophoresis. The DNA fragment, purified by use of a QIAEX gel extraction kit (QIAGEN CO., LTD.), was cleaved with restriction enzyme SalI in advance and then inserted into dephosphorylated plasmid pUC19 (MBI CO., LTD.) by use of a DNA ligation kit ver. 2 (TAKARA SHUZO CO., LTD.). Escherichia coli DH 5 α (GIBCO BRL CO., LTD.) was transformed by use of the pUC19 containing obtained DNA fragment. The obtained transformants was named pUC19hOBM. After proliferating the transformant, about 2.2 kb of human-OBMcDNA-inserted plasmids were purified therefrom in accordance with a commonly used method.

(5) Determination of the Nucleotide Sequence of cDNA Encoding the Full Length Amino Acid Sequence of Human OBM

The nucleotide sequence of the human OBMcDNA obtained in Example 19-(4) was determined by use of a Taq Dideoxy Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.). That is, the nucleotide sequence of the inserted fragment was determined by use of pUC19hOBM as a template. M13 Primer M3, M13 Primer RV (TAKARA SHUZO CO., LTD.), and a synthetic primer human OBM #8 designed based on the nucleotide sequence of the human OBMcDNA fragment (about 690 bp) were used as primers for determining the nucleotide sequence of the inserted fragment DNA of pUC19. The sequences of the primers, M13 Primer M3 and M13 Primer RV, are shown in SEQ ID NO 4 and SEQ ID NO 5 in the SEQUENCE LISTING, respectively. The amino acid sequence of human OBM estimated from the nucleotide sequence of the human OBMcDNA is shown in SEQ ID NO 11 in the SEQUENCE LISTING, and the nucleotide sequence of the human OBMcDNA is shown in SEQ ID NO 12 in the SEQUENCE LISTING.

The obtained plasmid containing the human OBMcDNA and the obtained Escherichia coli transformed by pUC19hOBM were deposited with the National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry with the deposition number FERM BP-6058 on August 13, 1997.

[Example 20]

¹²⁵I Labeling of OCIF and Quantitative Determination of ¹²⁵I-Labeled OCIF by ELISA

OCIF was ^{125}I -labeled in accordance with Iodogen method. 20 μl of 2.5 mg/ml Iodogen-chloroform solution was transferred to a 1.5 ml Eppendorf tube, and chloroform was evaporated at 40°C so as to prepare an Iodogen-coated tube.

5 After the tube was washed with 400 μl of 0.5 M sodium phosphate buffer (Na-Pi, pH: 7.0) three times, 5 μl of 0.5 M Na-Pi with a pH of 7.0 was added. Immediately after 1.3 μl (18.5 MBq) of Na- ^{125}I solution (Amersham Co., Ltd., NEZ-033H) was added to the tube, 10 μl of 1 mg/ml OCIF solution (monomer type or dimer type) was added. The resulting solution was mixed by means of a vortex mixer and left to stand at room temperature for 30 seconds. The solution was transferred to a tube containing 80 μl of 0.5 M Na-Pi solution (pH: 7.0) containing 10 mg/ml potassium iodide and 5 μl of phosphate buffered saline containing 5% bovine serum albumin (BSA-PBS), and mixed. The solution was added to a spin column (1 ml, G-25 Sephadex fine, Pharmacia Co., Ltd.) equilibrated with BSA-PBS and centrifuged at 2,000 rpm for 5 minutes. After 400 μl of BSA-PBS was added to a fraction eluted from the column and the fraction was mixed, 2 μl of the each fraction was sampled, and the radioactivity of the sample was measured by means of a gamma counter. The radiochemical purity of the prepared ^{125}I labeled OCIF solution was determined by measuring the radioactivity of a fraction precipitated by addition 10% trichloroacetic acid TCA.

The OCIF biological activity of the ^{125}I labeled OCIF was measured in accordance with a method described in WO96/26217. Further, the concentration of ^{125}I labeled OCIF was measured by ELISA in the following manner. That is, 100 μl of 50 mM NaHCO_3 (pH: 9.6), having 2 $\mu\text{g}/\text{ml}$ of rabbit anti-OCIF polyclonal antibody described in WO96/26217 dissolved therein, was added to each well of 96 well immunoplate (Nunc Co., Ltd., MaxiSorpTM) and left to stand at 4°C overnight. After this solution was discarded, 200 μl of mix-solution of BLOCKACE (Snow Brand Milk Products Co., Ltd.) and a phosphate buffered saline (mixing ratio = 25:75) (B-PBS) was added to each well and then left to stand at room temperature for 2 hours. After

the solution was discarded, each well was washed with a phosphate buffered saline containing 0.01% Polysorbate 80 (P-PBS) three times. Thereafter, 100 µl of B-PBS containing a ¹²⁵I labeled OCIF sample or OCIF reference standard was added to each well and left to stand at room temperature for 2 hours. After the solution was discarded, each well was washed with 200 µl of P-PBS six times. Then, 100 µl of diluted solution of peroxidase-labeled anti-OCIF rabbit polyclonal antibody in B-PBS was added to each well and left to stand at room temperature for 2 hours. After the solution was discarded, each well was washed with 200 µl of P-PBS six times. Then, 100 µl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well and then left to stand at room temperature for 2 to 3 minutes. Thereafter, 100 µl of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at 450 nm was measured by means of a microplate reader. The concentration of the ¹²⁵I labeled OCIF was determined from a calibration curve prepared by use of the OCIF reference standard.

[Example 21]

Expression of Protein Encoded by the cDNA of the Present Invention

(1) Construction of hOBM Expression Vector for Animal Cell

pUChOBM was cleaved with a restriction enzyme SalI, about 2.2 kb DNA fragments were purified by 1% agarose electrophoresis, and blunt-ended with DNA Blunting Kit (TAKARA SHUZO CO., LTD.) (the resulted DNA fragment with smoothed terminals is called "smoothed hOBMcDNA fragment"). Expression plasmid pcDL-SR α296 (Molecular and Cellular Biology, Vol. 8, pp. 466 to 472 (1988)) was cleaved with a restriction enzyme EcoRI, and blunt-ended with the blunting kit. The resulted expression plasmid was bound to the smoothed hOBMcDNA fragment by use of a DNA ligation kit ver. 2. Using the ligation reaction solution, Escherichia coli DHα was transformed. From the obtained ampicillin-resistant transformant, a clone, having a phOBM plasmid in which hOBMcDNA inserted with forward direction for transcription direction of SRα promoter, was selected by analysis of DNA map obtained by restriction enzyme

cleavage and determination of DNA sequences. The obtained clone was named DH5 α /phOBM.

(2) Expression of Human OBM in COS-7 Cell

E coli, DH5 α /phOBM, was cultured and the plasmid
5 phOBM was purified with QIA Filter Plasmid Midi Kit (QIAGEN CO., LTD.) . The phOBM was transfected into COS-7 cells in each well of 6 well plate by use of lipofectamine, and the cells were cultured in DMEM containing 10% fetal bovine serum for 2 days. The medium was replaced with cysteine-methionine-
10 free DMEM (DAINIPPON PHARMACEUTICAL CO., LTD.) containing 5% dialyzed fetal bovine serum (88 μ l/well), and the cells were cultured for another 15 minutes. Then, 14 μ l of Express Protein Labeling Mix (NEN CO., LTD., 10 mCi/ml) was added. After the cells were cultured for 4 hours, 200 μ l of DMEM
15 containing 10% fetal bovine serum was added, and the cells were cultured for 1 hour. After the cells were washed with PBS twice, 0.5 ml of TSA buffer (10 mM Tris-HCl (pH: 8.0) containing 0.14 M NaCl and 0.025% NaN₃) containing 1% Triton X-100, 1% bovine hemoglobin, 10 μ g/ml leupeptin, 0.2 TIU/ml
20 aprotinin and 1 mM PMSF was added, and the cells were left to stand on ice for 1 hour. After the cells were crushed by pipetting, the resulted lysate was centrifuged at 4°C and 3,000 X g for 10 minutes so as to obtain a supernatant. 200 μ l of dilution buffer (TSA buffer containing 0.1% Triton X-100,
25 0.1% bovine hemoglobin, 10 μ g/ml leupeptin, 0.2 TIU/ml aprotinin and 1 mM PMSF) was added to 100 μ l of the supernatant, , and the resulting supernatant was shaken together with Protein A Sepharose (50 μ l) at 4°C for 1 hour. Thereafter, the solution was centrifuged at 4°C, 1,500 X g for
30 1 minute so as to collect a supernatant. Thereby, a protein non-specifically binding the Protein A Sepharose was removed. OCIF (1 μ g) was added to the supernatant, and the resulting supernatant was shaken at 4°C for 1 hour so as to bind OBM and OCIF together. Then, an anti-OCIF rabbit polyclonal antibody
35 (50 μ g) was added, and the resulting solution was shaken at 4°C for 1 hour. Then, protein A Sepharose (10 μ l) was added to the solution and the solution was then shaken at 4°C for 1 hour.

The solution was centrifuged at 4°C, 1,500 X g for 1 minute so as to collect a precipitated fraction. The precipitate resulting from the centrifugation was washed with the dilution buffer twice, with a bovine hemoglobin free dilution buffer twice, with a TSA buffer once, and with 50 mM Tris-HCl (pH: 6.5) once. After washing, an SDS buffer (0.125 M Tris-HCl, 4% dodecyl sodium sulfate, 20% glycerol, 0.002% bromophenol blue, pH: 6.8) containing 10% β mercaptoethanol was added to the precipitate. The precipitate was heated at 100°C for 5 minutes and it was subjected to SDS-PAGE (12.5% polyacrylamide gel, DAIICHI KAGAKU CO., LTD.). The gel was fixed and dried in accordance with a commonly used method and the signals of isotopes from the fixed gel were amplified by Amplify (Amersham Co., Ltd.), and the fixed gel was exposed to Bio Max MR Film (KODAK CO., LTD.) at -80°C. The results are shown in Fig. 8. As a result, it was revealed that the molecular weight of protein encoded by the cDNA of the present invention was about 40,000.

[Example 22]

Binding of Protein Encoded by the cDNA of the Present Invention to OCIF

In the same manner as in Example 21-(2), the purified phOBM was transfected into COS-7 cells in each well of 24 well plate by use of lipofectamine, and the cells were cultured for 2 or 3 days. Then, the cells were washed with serum-free DMEM, and 200 μ l of medium for binding assay (serum-free DMEM containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.1 mg/ml heparin and 0.2% NaN_3), containing 20 ng/ml of ^{125}I labeled OCIF, was added to some wells. Further, to other wells, 200 μ l of the medium for binding assay, containing 8 μ g/ml of unlabeled OCIF in addition to 20 ng/ml of the ^{125}I labeled OCIF, was added so as to conduct following experiments. After cultured in a CO_2 incubator (5% CO_2) at 37°C for 1 hour, the cells were washed twice with 500 μ l of phosphate buffered saline containing 0.1 mg/ml heparin. After washing, 500 μ l of 0.1 N NaOH solution was added to each well, and the wells were then left to stand at room temperature for 10 minutes so as to dissolve the cells. The amount of ^{125}I in

each well was measured by means of a gamma counter. As a result, it was confirmed that the ^{125}I labeled OCIF bound only to a cell transfected with phOBM as shown in Fig. 19.

Further, it was also confirmed that the binding was

significantly inhibited by addition of unlabeled OCIF (8 $\mu\text{g}/\text{ml}$) of 400-fold concentration. From these results, it was revealed that a protein human OBM coded by a cDNA on phOBM specifically bound to OCIF on the surface of a COS-7 cell.

[Example 23]

Crosslinking Experiment of ^{125}I Labeled OCIF to Protein encoded by the cDNA of the Present Invention

To further analyze the characteristics of the protein encoded by the cDNA of the present invention, crosslinking of ^{125}I labeled monomer type OCIF with the protein encoded by the cDNA of the present invention was conducted. That is, after expression vectors phOBM were prepared and transfected into COS-7 cells in accordance with the methods described in Examples 21-(1) and (2), 200 μl of the medium for the binding assay containing the ^{125}I labeled OCIF (25 ng/ml) was added to some wells. Further, the medium for binding assay containing unlabeled OCIF of 400-fold concentration in addition to the ^{125}I labeled OCIF was added to other wells. The cells were cultured in a CO_2 incubator (5% CO_2) at 37°C for 1 hour, the cells were washed twice with 500 μl of phosphate buffered saline containing 0.1 mg/ml of heparin. To these cells, 500 μl of phosphate buffered saline dissolving 100 $\mu\text{g}/\text{ml}$ of crosslinking agent DSS (Disuccinimidyl suberate, Pierce Co., Ltd.) was added, and the cells incubated at 0°C for 10 minutes for reaction. After the cells in these wells were washed twice with 1 ml of phosphate buffered saline cooled to 0°C , 100 μl of 20 mM Hepes buffer containing 1% Triton X-100 (Wako Pure Chemical Industries, Ltd.), 2 mM PMSF (phenylmethylsulfonyl fluoride, sigma Co., Ltd.), 10 μM pepstatin (Wako Pure Chemical Industries, Ltd.), 10 μM leupeptin (Wako Pure Chemical Industries, Ltd.), 10 μM antipain (Wako Pure Chemical Industries, Ltd.) and 2 mM EDTA (Wako Pure Chemical Industries, Ltd.) was added to these cells, and the

wells were left to stand at room temperature for 30 minute so as to lise the cells. After 15 µl of these samples were treated with SDS under nonreducing conditions in accordance with a commonly used method, the samples were subjected to electrophoresis with a gel for SDS electrophoresis (4 to 20% polyacrylamide gradient, DAIICHI KAGAKU CO., LTD.). After the electrophoresis, the gel was dried and exposed to BioMAX MS Film (Kodak Co., Ltd.) with BioMax MS Intensifying amplifying Screen (Kodak Co., Ltd.) at -80°C for 24 hours. The exposed films were developed in accordance with a commonly used method. As a result, a protein band having a molecular weight of about 90,000 to 110,000 was detected as shown in Fig. 20 in crosslinking between ¹²⁵I labeled monomer type OCIF and the protein encoded by the cDNA of the present invention..

[Example 24]

Expression of Secretory-Type Human OBM

(1) Construction of Secretory-Type Human OBM Expressing Plasmid

A PCR reaction was carried out by use of human OBM SF (SEQ ID NO 13 in the SEQUENCE LISTING) and mouse OBM #8 (SEQ ID NO 6 in the SEQUENCE LISTING) as primers and pUC19hOBM as a template. After the product was purified by agarose gel electrophoresis, it was cleaved with restriction enzymes SP1I and HindIII and then purified by agarose gel electrophoresis so as to obtain 0.27 kb fragment. A fragment of hOBMcDNA which was cleaved at only one site of restriction enzyme DraI by partial cleavage of human OBM cDNA therewith, and purified by agarose gel electrophoresis, and the purified fragment was further cleaved with a restriction enzyme HindIII. 0.53 kb of DraI/HindIII fragment was purified by agarose gel electrophoresis, and the purified fragment and the Sp1I/HindIII fragment (0.27 kb) of the above PCR product together with an SP1I/EcoRV fragment (5.2 kb) of pSec TagA (InVitrogen Co., Ltd.) were subjected to ligation by use of a ligation kit ver. 2 (TAKARA SHUZO CO., LTD.), and Escherichia coli DH5α were transformed by use of the reaction product of ligation. Plasmids were purified from the obtained ampicillin-resistant clone by alkaline SDS method and cleaved by restriction enzymes so as to select a plasmid having 0.27

kb and 0.53 kb of fragments inserted in pSec TagA. The plasmid was subjected to sequencing by use of a Taq Dideoxy Terminator Cycle Sequencing FS Kit (Perkin Elmer Co., Ltd.), thereby confirming that the plasmid had sequences encoding secretory-type human OBM. After the plasmid was cleaved by restriction enzymes NheI and XhoI, a fragment (0.8 kb) corresponding to secretory-type human OBM cDNA was collected by agarose gel electrophoresis. This fragment was inserted into an NheI/XhoI fragment (10.4 kb) of an expression vector pCEP4 (InVitrogen Co., Ltd.) by use of the ligation kit, and Escherichia coli DH5 α were transformed by use of the reaction product of the ligation. Plasmids were purified from the obtained ampicillin-resistant clones by alkaline SDS method and cleaved by restriction enzymes so as to select a Escherichia coli clone having a secretory-type human OBM expression plasmid (pCEPshOBM) with a target structure. The Escherichia coli clone having the pCEPshOBM was cultured, and the pCEPshOBM was purified by use of QIA Filter Plasmid Midi Kit (QIAGEN CO., LTD.).

(2) Expression of Secretory-Type OBM

293-EBNA cells were suspended in IMDM containing 10% FCS (IMDM-10%FCS), seeded in a collagen-coated 24 well plate (SUMITOMO BAKELITE CO., LTD.) in an amount of 2×10^5 cells/2 ml/well, and cultured overnight. To the cells, 1 μ g of pCEPshOBM and pCEP4 was transduced by use of 4 μ l of lipofectamine (GIBCO CO., LTD.), and the cells were cultured for another 2 days in 0.5 ml of serum-free IMDM or IMDM-10%FCS, thereby collecting a conditioned medium. Expression of secretory-type human OBM in the conditioned medium was confirmed in the following manner. That is, sodium hydrogen carbonate was added to the conditioned medium to a final concentration of 0.1 M and left to stand at 4°C overnight, and the human OBM in the conditioned medium was solid-phased in a 96 well plate. BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution diluted to 4 times with PBS (B-PBS) was added to each wells and the plate was left to stand at room temperature for 2 hours so as to cause blocking. 3-100 ng/ml of OCIF diluted with B-PBS was added to the wells and left to stand at 37°C

for 2 hours. After the plate was washed with PBS containing 0.05% Polysorbate 20 (P-PBS), 100 µl of peroxidase labeled anti-OCIF antibody described in WO96/26217 diluted with B-PBS was added to each well and left to stand at 37°C for 2 hours.

After each well was washed with P-PBS six times, 100 µl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well and then left to stand at room temperature for about 10 minutes. Thereafter, 100 µl of Stopping Reagent (Scytek Co., Ltd.) was added to each well.

The absorbance of each well at 450 nm was measured by means of a microplate reader. The results are shown in Fig. 21. In the plate having the solid-phased conditioned medium of the cells transduced with the pCEPshOBM, absorption at 450 nm increased depending on the concentration of the OCIF added.

Meanwhile, in the case where the conditioned medium of the cells transduced only with the vector pCEP4 was solid-phased, no increase in absorption was seen. Further, Fig. 22 shows the results of an experiment in which the proportion of the conditioned medium used for solid phasing was varied within a range of 5 to 90% and a certain concentration of OCIF (50 ng/ml) was added. In the plate having the solid-phased conditioned medium of the cells transduced with the pCEPshOBM, absorption at 450 nm increased along with an increase in the proportion of the conditioned medium added. Meanwhile, in the plate having the solid-phased conditioned medium of the cells transduced with the vector pCEP4, no increase in absorption was observed. From these results, it was confirmed that secretory-type human OBM was expressed in the conditioned medium of the cells transduced with the pCEPshOBM.

[Example 25]

Expression of Thioredoxin-Human OBM Fusion Protein (Trx-hOBM)

(1) Construction of Thioredoxin-Human OBM Fusion Protein (Trx-hOBM) Expression Vector

10 µl of 10X ExTaq buffer (TAKARA SHUZO CO., LTD.), 8 µl of 10 mM dNTP (TAKARA SHUZO CO., LTD.), 77.5 µl of sterilized distilled water, 2 µl of pUC19hOBM aqueous solution (10 ng/µl), 1 µl of primer mouse OBM #3 (SEQ ID NO 9 in the SEQUENCE LISTING) (100 pmol/µl), 1 µl of primer hOBM SalR2 (SEQ

ID NO 14 in the SEQUENCE LISTING) (100 pmol/ μ l) and 0.5 μ l of ExTaq (5 μ l/ μ l) (TAKARA SHUZO CO., LTD.) were mixed together in a micro centrifuging tube so as to cause a PCR reaction. After the reaction consisting of at 95°C for 5 minutes, 50°C for 1 second, 55°C for 1 minute, 74°C for 1 second and 72°C for 5 minutes, the cycle reaction consisting of at 96°C for 1 minute, 50°C for 1 second, 55°C for 1 minute, 74°C for 1 second and 72°C for 3 minutes, was repeated 25 times. About 750 bp of DNA fragment was purified from the whole reaction solution. After the purified DNA fragment (whole) was cleaved with restriction enzymes Sali (TAKARA SHUZO CO., LTD.) and BspHI (NEW ENGLAND BILABS CO., LTD.), 1% agarose gel electrophoretic migration was carried out so as to purify about 320 bp of DNA fragment (fragment 1) and dissolve the fragment in 20 μ l of sterilized distilled water. Similarly, about 450 bp of DNA fragment (fragment 2) which is a cleaved product of 4 μ g of pUC19hOBM described in Example 19-(3) by a restriction enzyme BamHI, BspHI (TAKARA SHUZO CO., LTD.) and about 3.6 kb of DNA fragment (fragment 3) which is a cleaved product of 2 μ g of pTrxFus (InVitrogen Co., Ltd.) by a restriction enzyme BamHI, Sali (TAKARA SHUZO CO., LTD.) were purified and then dissolved in 20 μ l of sterilized distilled water. To purify the DNA fragments, a QIAEX II gel extraction kit was used. Fragment 1, 2 and 3 were combined by use of a DNA ligation kit ver. 2 (TAKARA SHUZO CO., LTD.) by keeping them at 16°C for 2.5 hours. Escherichia coli GI724 strains (InVitrogen Co., Ltd.) was transformed using the ligation reaction solution, in accordance with a method described in an instruction manual attached to a ThioFusion Expression System (InVitrogen Co., Ltd.). From the obtained ampicillin-resistant transformants, a clone, having a plasmid in which an hOBMcDNA fragment was bound to a thioredoxin gene in the same reading frame, was selected by analysis of DNA map obtained by restriction enzyme cleavage and determination of DNA sequences. The obtained strain was named GI724/pTrxhOBM25.

(2) Expression of Trx-OBM in Escherichia coli

GI724/pTrxhOBM strain and GI724 strain having pTrxFus (GI724/pTrxFus) were shaking-cultured at 37°C for 6 hours in 2

ml of RMG-Amp medium (0.6% Na_2HPO_4 , 0.3% KH_2PO_4 , 0.05% NaCl , 0.1% NH_4Cl , 2% casamino acid, 1% glycerol, 1 mM MgCl_2 , 100 $\mu\text{g/ml}$ ampicillin, pH: 7.4). 0.5 ml of the culture suspension was added to 50 ml of Induction medium (0.6% Na_2HPO_4 , 0.3% KH_2PO_4 , 0.05% NaCl , 0.1% NH_4Cl , 0.2% casamino acid, 0.5% glucose, 1 mM MgCl_2 , 100 $\mu\text{g/ml}$ ampicillin, pH: 7.4) and shaking-cultured at 30°C. L-tryptophan was added so as to achieve a final concentration of 0.1 mg/ml when the value at $\text{OD}_{600 \text{ nm}}$ became about 0.5, and the cells were further shaking-cultured at 30°C for another 6 hours. The culture suspension was centrifuged at 3,000 X g so as to collect cells, and then the collected cell was suspended in 12.5 ml of PBS. The suspension was subjected to an ultrasonic generator (Ultrasonics Co., Ltd.) so as to crush the cells and then the sample was centrifuged at 7,000 X g for 30 minutes so as to collect a supernatant as a soluble protein fraction. 10 μl of the fraction solution was subjected to SDS-PAGE (10% polyacrylamide) under reducing conditions. As a result, as shown in Fig. 23, a protein band having a molecular weight of about 40,000, which could not be seen in the soluble protein fraction of GI724/pTrxFus, was detected in the soluble protein fraction of GI724/pTrxOBM. From the above results, it was confirmed that a thioredoxin-human OBM fusion protein (Trx-OBM) was expressed in the Echerichia coli clone.

(3) Binding Ability of Trx-hOBM to OCIF

It was confirmed by the following experiment that the expressed Trx-hOBM bound to OCIF. That is, 100 μl of anti-thioredoxin antibody (InVitrogen Co., Ltd.) diluted to be 1/5,000 with 10 mM sodium hydrogen carbonate aqueous solution was added to each well of 96 well immunoplate (Nunc Co., Ltd.) and the plate was left to stand at 4°C overnight. After the solution in each cell was discarded, 200 μl of a solution obtained by diluting BLOCKACE (Snow Brand Milk Products Co., Ltd.) to be 1/2 with PBS (BA-PBS) was added to each well and then the plate was left to stand at room temperature for 1 hour. After the solution was discarded, each well was washed with P-PBS three times. 100 μl of the GI724/pTrxOBM-derived soluble protein fraction solution diluted stepwise with BA-PBS

and 100 µl of the GI724/pTrxFus-derived soluble protein fraction solution diluted stepwise with BA-PBS were added to each well and the plate was left to stand at room temperature for 2 hours. After each well was washed with P-PBS three times, 100 µl of OCIF (100 ng/ml) diluted with BA-PBS was added to each well and the plate was left to stand at room temperature for 2 hours. After each well was washed with P-PBS three times, 100 µl of peroxidase labeled anti-OCIF antibody described in W096/26217 diluted to be 1/2,000 with BA-PBS was added to each well and the plate was left to stand at room temperature for 2 hours. After each well was washed with P-PBS six times, 100 µl of TMB solution was added to each well and then the plate was left to stand at room temperature for about 10 minutes. Thereafter, 100 µl of Stopping Reagent was added to each well. The absorbance of each well at 450 nm was measured by means of a microplate reader. The results are shown in Fig. 24. No difference was observed between absorbance resulted in presence and absence of the GI724/pTrxFus-derived soluble protein fraction solution, while with the GI724/pTrxhOBM-derived soluble protein fraction solution, the absorbance increased depending on an increase in the concentration of the GI724/pTrxOBM derived soluble protein fraction solution. Further, Fig. 25 shows the results of an experiment in which the dilution rate of the soluble protein fraction solution to be added was kept constant (1% concentration) and OCIF (0-100 ng/ml) diluted stepwise with BA-PBS was added. For the GI724/pTrxFus-derived soluble protein fraction solution, absorbance remained low regardless of the concentration of OCIF, while for the GI724/pTrxhOBM-derived soluble protein fraction solution, absorbance increased in OCIF concentration-dependent manner. It was confirmed from this result that Trx-hOBM produced in GI724/pTrxhOBM had an ability to bind OCIF.

(4) Large Scale Culture of Escherichia coli Producing Trx-hOBM

GI724/pTrxhOBM was spread on an RMG-Amp agar medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 2% casamino acid, 1.5% agar, pH: 7.4) with a platinum loop and cultured at 30°C overnight. The cells were suspended in 10 ml of

Induction medium, and every 5 ml of the suspension was added to each of two conical flasks of 2L volume containing 500 ml of Induction medium, and the flasks were shaking-cultured at 30°C. L-tryptophan was added so as to achieve a final concentration of 0.1 mg/ml when absorbance at OD_{600 nm} became about 0.5, and the shaking culture at 30°C was continued for another 6 hours. The culture suspension was centrifuged at 3,000 X g for 20 minutes so as to collect cell, and the collected cell was then suspended in 160 ml of PBS. The suspension was subjected to ultrasocination (Ultrasonics Co., Ltd.) so as to crush the cell and the cell lysate was then centrifuged at 7,000 X g for 30 minutes so as to collect a supernatant as a soluble protein fraction.

(5) Preparation of OCIF-Immobilized Affinity Column

2 g of TSKgel AF-Tresyl TOYOPAL 650 (TOSO CO., LTD.) and 40 ml of 1.0 M potassium phosphate buffer (pH: 7.5) containing 35.0 mg of recombinant OCIF prepared by a method described in W096/26217 were mixed together and gently shaken at 4°C overnight so as to cause a coupling reaction. To inactivate excessive active residue, after a supernatant was removed by centrifugation, 40 ml of 0.1 M Tris-HCl buffer (pH: 7.5) was added to a precipitated carrier, and the mixture was gently shaken at room temperature for 1 hour. After 0.1 M glycine-HCl buffer containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH: 3.3) and a 0.1 M sodium citrate buffer containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH: 2.0) were passed through a column (in which the obtained gel was packed) so as to wash it, the column was washed twice with 10 mM sodium phosphate buffer containing 0.01% Polysorbate 80 (pH: 7.4) so as to equilibrate it.

(6) Purification of Trx-hOBM by OCIF-Immobilized Affinity Column

Purification of Trx-hOBM was carried out at 4°C unless otherwise stated. After the above-mentioned OCIF-immobilized affinity carrier (10 ml) and the above-mentioned soluble protein fraction solution (120 ml) described in Example 25-(4) were mixed together, the mixture was gently shaken at 4°C overnight in four of 50 ml centrifuging tubes by use of a rotor. The carrier in the mixture was filled in Econocolumn

(internal diameter: 1.5 cm, length: 15 cm, Biorad Co., Ltd.). 300 ml of PBS containing 0.01% Polysorbate 80, 100 ml of 10 mM phosphate buffer containing 0.01% Polysorbate 80 and 2.0 M NaCl (pH: 7.0), and 100 ml of 0.1 M glycine-HCl buffer containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH: 3.3) were passed through the column in turn so as to wash the column. Then, 0.1 M sodium citrate buffer containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH: 2.0) was passed through the column so as to elute proteins adsorbed to the column. Every 5 ml of eluates were fractionated. To the fractions, 10% volume of 2M Tris solution (pH: 8.0) was added so as to immediately neutralize the fractions. The presence or absence of Trx-hOBM in the each fraction of the eluate was examined in accordance with the method described in Example 25-(3). Fractions containing Trx-hOBM were collected and purified further.

(7) Purification of Trx-hOBM by Gel Filtration

About 25 ml of the Trx-hOBM fraction described in Example 25-(6) was centrifugal-concentrated to about 0.5 ml by use of Centriplus 10 and Centricon 10 (Amicon Co., Ltd.). The concentrated sample was subjected to a Superose 12 HR 10/30 column (1.0 X 30 cm, Pharmacia Co., Ltd.) equilibrated in advance with PBS containing 0.01% Polysorbate 80. The column was developed at a flow rate of 0.25 ml/min by using PBS containing 0.01% Polysorbate 80 as a mobile phase so as to fractionate every 0.25 ml of eluates from the column. Trx-OBM in the fractions was detected by the method described in Example 25-(3) and SDS-PAGE. Fractions containing purified Trx-hOBM were collected so as to measure the protein concentration of Trx-OBM. The protein concentration was measured with DC-protein assay kit (Biorad Co., Ltd.) using bovine serum albumin as a reference standard.

[Example 26]

Osteoclastogenesis Inducing Activity of OBM

phOBM and pcDL-SR α 296 were transfected into COS-7 cells by use of lipofectamine (GIBCO CO., LTD.), respectively. After the cells were cultured in DMEM containing 10% FCS for 1 day, they were trypsinized and seeded in a 24 well of plate in which cover glass (15 mm round, MATSUNAMI CO., LTD.) was seated at a concentration of 5×10^4 cells/well, and then

cultured for another two days. The cultured plate was washed with PBS once and then PBS containing 1% paraformaldehyde was added, and the cells were incubated at room temperature for 8 minutes so as to fix the cells on the cover glass. After the plate having fixed cell was washed with PBS six times, 700 μ l of mouse spleen cells suspended in α -MEM containing 10^{-8} M activated vitamin D₃, 10^{-7} M dexamethasone and 10% fetal bovine serum in an amount of 1×10^6 cells/ml was added to each well. Millicell PCF (Millipore Co., Ltd.) was set on each well, and 700 μ l of ST2 cells suspended in the above medium in a concentration of 4×10^4 cells/ml were added to the Millicell PCF and cultured at 37°C for 6 days. After that, the Millicell PCF was removed and the plate was washed with PBS once. Then, the cell was fixed for a minute by an acetone-ethanol solution (50:50) and a cell, having tartaric acid resistant acid phosphatase activity (TRAP activity), which is specific marker of osteoclast, were stained by use of a LEUKOCYTE ACID PHOSPHATASE kit (sigma Co., Ltd.). As a result of observation under the microscope, cell having TRAP activity was not detected in the wells having COS-7 cells transfected with the pcDL-SR α 296, while 65 ± 18 (n = 3, average \pm standard deviation) of TRAP positive cells were observed in the wells having cells transfected with phOBM. Further, it was also confirmed that these TRAP positive cells expressed calcitonin receptors, since the cells showed specific binding to ¹²⁵I labeled salmon calcitonin (AMSHAM CO., LTD.). From these results, it was revealed that human OBM, a protein encoded by the cDNA of the present invention, had an activity to promote osteoclast formation.

[Example 27]

Osteoclastogenesis Promoting Activities of Trx-hOBM and Secretory-Type human OBM

Mouse spleen cells were suspended in α -MEM containing 10^{-8} M activated vitamin D₃, 10^{-7} M dexamethasone and 10% fetal bovine serum at a concentration of 2×10^6 cells/ml, and 350 μ l of the suspension was added to each well of a 24 well plate. After 350 μ l of a solution prepared by diluting purified Trx-

OBM, (40 ng/ml) with the above medium, or, 350 µl of a solution prepared by diluting a conditioned medium obtained when 293-EBNA cells transduced by pCEPshOBM or pCEP4 were cultured in IMDM-10%FCS to be 1/10 with the above medium, or, 350 µl of the above medium alone was added, Millicell PCF (Millipore Co., Ltd.) was set on each well, and 600 µl of ST2 cell suspension in the above medium at a concentration of 4×10^4 cells/ml were added to the Millicell PCF. After the cell were cultured for 6 days, the Millicell PCF was removed, and the plate was washed with PBS once. Then, after the cells were fixed for 1 minute by an acetone-ethanol solution (50:50), cells having tartaric acid resistant acid phosphatase activity (TRAP activity) were stained by use of a LEUKOCYTE ACID PHOSPHATASE kit (sigma Co., Ltd.). As a result of observation under the microscope, cells having TRAP activity was not detected in the wells containing no Trx-hOBM, while 115 ± 19 ($n = 3$, average \pm standard deviation) of TRAP positive cells were observed in the wells containing Trx-hOBM. Similarly, cells having TRAP activity was not detected in the wells containing the conditioned medium of pCEP4-transduced 293-EBNA, while 125 ± 23 ($n = 3$, average \pm standard deviation) of TRAP positive cells were observed in the wells containing the conditioned medium of pCEPshOBM-transduced 293-EBNA. Furthermore, it was also confirmed that these TRAP positive cells expressed calcitonin receptors, since the cells showed specific binding to ^{125}I labeled salmon calcitonin (AMSHAM CO., LTD.). From these results, it was revealed that Trx-hOBM and secretory-type OBM had an activity to promote osteoclast formation.

[Example 28]

Preparation of Polyclonal Antibody

Mouse sOBM or human sOBM, which was used as an immunizing antigen, was obtained in accordance with the above-mentioned method. That is, mouse sOBM cDNA (cDNA which codes mouse sOBM (SEQ ID NO 16 in the SEQUENCE LISTING) having no membrane binding site with lacking amino acids region between the amino acid of N terminal end and the 72nd amino acid from N terminal end of mouse OBM, SEQ ID NO 18 in the SEQUENCE LISTING) or human OBM cDNA (cDNA which codes human sOBM (SEQ

ID NO 17 in the SEQUENCE LISTING) having no membrane binding site with lacking amino acids region between the N terminal end and the 71st amino acid from N terminal end of human OBM, SEQ ID NO 19 in the SEQUENCE LISTING), together with a Hind III/EcoR V fragment (5.2 kb) of a pSec TagA expression vector (InVitrogen Co., Ltd.), containing nucleotide sequence coding a signal peptide of κ -chain of immunoglobulin, and an EcoRI/PmaCI fragment (0.32 kb) of OBM cDNA, were subjected to ligation by use of a ligation kit ver. 2 (TAKARA SHUZO CO., LTD.), and *Escherichia coli* DH5 α were transformed with the reaction product,. Plasmids were purified from the obtained ampicillin-resistant clones by alkaline SDS method and cleaved by restriction enzymes so as to select a plasmid having 0.6 kb and 0.32 kb of fragments inserted in pSec TagA. As a result of determining the sequences of the plasmid by use of Dye Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.), it was confirmed that the plasmid had sequences encoding mouse or human sOBM. The plasmid was cleaved by restriction enzymes NheI and XhoI and then a fragment (1.0 kb) corresponding to secretory-type OBM cDNA was collected by agarose gel electrophoresis. The fragment was inserted into an NheI/XhoI fragment (10.4 kb) of an expression vector pCEP4 (InVitrogen Co., Ltd.) by use of a ligation kit, and , *Escherichia coli* DH5 α were transformed by use of the reaction product. Plasmids were purified from the obtained ampicillin-resistant clones by an alkaline SDS method and cleaved by restriction enzymes and analyzed so as to select a *Escherichia coli* sclone having a secretory OBM expression plasmid (pCEP sOBM) having a target structure. The *Echerichia coli* clone having the pCEP sOBM was cultured, and the pCEP sOBM was purified by use of a QIA Filter Plasmid Midi Kit (QIAGEN CO., LTD.). Next, 293-EBNA cell was suspended in IMDM containing 10% FCS (IMDM-10%FCS) and seeded in a collagen-coated 24 well plate (SUMITOMO BAKELITE CO., LTD.) in an amount of 2×10^5 cells/2 ml/well, and cultured overnight. To the cells, 1 μ g of pCEP sOBM or pCEP4 was transduced by use of 4 μ l of lipofectamine (GIBCO CO., LTD.), and the cells were cultured for another 2 days in 0.5 ml of serum-free IMDM or IMDM-10%FCS, thereby collecting a

conditioned medium. The clones which highly produced recombinant mouse soluble OBM (msOBM) or human soluble OBM (hsOBM) were screened in the following manner. After sodium hydrogen carbonate was added to the conditioned medium seemed to contain msOBM or hsOBM at a final concentration of 0.1 M, 100 µl of the conditioned medium was added to each well of 96 well immunoplate (Nunc Co., Ltd.) and the plate was left to stand at 4°C overnight so as to solid-phase the msOBM or hsOBM in the conditioned medium on each well. Then, 200 µl of BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution diluted to 4 times with PBS (B-PBS) was added to each well of the plate and the plate was left to stand at room temperature for 2 hours. After three times washing with PBS containing 0.1% Polysorbate 20 (P-PBS), 100 µl of recombinant OCIF (rOCIF) solution diluted stepwise (0-100 ng/ml) with B-PBS was added to each well and the plate was left to stand at 37°C for 2 hours. After three times washing with PBS, 100 µl of peroxidase labeled anti-OCIF polyclonal antibody (WO96/26217) diluted with B-PBS was added to each well and the plate was left to stand at 37°C for 2 hours. After six times washing with P-PBS, 100 µl of TMB solution (TMB Soluble Agent, High sensitivity, Scytek Co., Ltd.) was added to each well and left to stand at room temperature for about 10 minutes. Thereafter, 100 µl of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at 450 nm was measured by means of a microplate reader. In the plate having the solid-phased protein derived from conditioned medium of the clone producing msOBM or hsOBM, the absorbance significantly increased in proportion to the concentration of the OCIF. As for the producing clones of msOBM or hsOBM, clone indicating high rate in increase of the absorbance were selected as highly producing clones thereof. Each of the highly producing clone of msOBM or hsOBM selected in above mentioned manner was mass-cultured by use of IMDM containing 5% FCS as a medium in 25 T-flasks (T-225). After the cell grown to confluent, 100 ml of fresh medium was added to each T-255 flask and the cell was further cultured for 3 or 4 days and then a conditioned medium was collected. By repeating

this procedure 4 times, 10 liters of the conditioned medium containing msOBM and 10 liters of the conditioned medium containing hsOBM were obtained. About 10 mg of purified msOBM and about 12 mg of purified hsOBM which were uniform (molecular weight: 32 kDa) in terms of SDS-polyacrylamide electrophoresis were obtained by carrying out purification on above-obtained conditioned medium with the affinity chromatography using an rOCIF-immobilized column and gel filtration chromatography in accordance with the method described in Examples 25-(6) and (7). The obtained purified samples were used as immunizing antigens. The obtained antigens each were dissolved in phosphate buffered saline (PBS) at a concentration of 200 µg/ml and then the solution mixed with an equal amount of Freund's complete adjuvant so as to be emulsified. 1 ml of each emulsion was subcutaneously administered to three Japanese white rabbits at an interval of about one week so as to immunize the rabbits. An antibody titer was measured, and when the antibody titer reached a maximum, a booster was carried out. 10 days after the booster, all blood was collected from all the rabbits. Antiserum was diluted to two times with binding buffer for protein A sepharose chromatography (BioRad Co., Ltd.) and then added to a protein A column equilibrated with the above buffer. After the column was efficiently washed with the above buffer, an anti-sOBM antibody adsorbed to the column was eluted by an elution buffer (BioRad Co., Ltd.) or 0.1 M glycine-HCl buffer (pH: 2.9 to 3.0). In order to neutralize the antibody-containing eluate immediately, the eluted solution was fractionated by use of a test tube containing a small amount of 1.0 M Tris-HCl (pH: 8.0). The antibody eluate was dialyzed in PBS at 4°C overnight. The amount of protein in the antibody solution was measured in accordance with a Lowry method using bovine IgG as a standard. Thus, the purified immunoglobulin (IgG) containing the polyclonal antibody of the present invention was obtained in an amount of about 10 mg per 1 ml of rabbit antiserum.

[Example 29]

Measurements of OBM and sOBM by ELISA Using Polyclonal Antibody

Sandwich ELISA, using the rabbit anti-hsOBM polyclonal antibody obtained in Example 28 as a solid phase antibody and as an enzyme labeled antibody, was constructed. As enzyme labeling, peroxidase (POD) labeling was carried out in accordance with a method of Ishikawa et al. (Ishikawa et al.: J. Immunoassay, Vol. 4, 209 to 327, 1983). The anti hsOBM polyclonal antibody obtained in Example 28 was dissolved in a 0.1 M NaHCO₃ solution at a concentration of 2 µg/ml, and 100 µl of the resulting solution was added to each well of 96-well immunoplate (Nunc Co., Ltd.) and the plate was left to stand at room temperature overnight. Then, 200 µl of 50% BLOCKACE (Snow Brand Milk Products Co., Ltd.) was added to each well and the plate was left to stand at room temperature for 1 hour, and each well was washed with PBS containing 0.1% polysorbate 20 (washing buffer) three times. The purified human OBM, which was expressed in the same manner as in Example 26 and was purified in the same manner as in Example 2, and the purified human sOBM, obtained in Example 28, was diluted stepwisely with primary reaction buffer (0.2 M Tris-HCl buffer containing 40% BLOCKACE and 0.1% polysorbate 20, pH: 7.2) and 100 µl of each diluent were added to each well. After the plate was left to stand at room temperature for 2 hours, each well was washed with the above washing buffer three times. (The purified human OBM, which was expressed in the same manner as in Example 26 and was purified in the same manner as in Example 2, and the purified human sOBM, obtained in Example 28, was diluted stepwisely with primary reaction buffer (0.2 M Tris-HCl buffer containing 40% BLOCKACE and 0.1% polysorbate 20, pH: 7.2) and 100 µl of each diluent were added to each well. After the plate was left to stand at room temperature for 2 hours, each well was washed with the above washing buffer three times. [Note: The underlined sentences are full refrain of just previous sentences, which are found in original Japanese-language specification of the international application.] 100 µl of POD labeled anti-human sOBM polyclonal antibody diluted to 1,000 times with secondary reaction buffer (0.1 M Tris-HCl buffer containing 25% BLOCKACE and 0.1% polysorbate 20, pH: 7.2) was added to each well and the plate

was left to stand at room temperature for 2 hours, and each well was washed with the washing buffer three times. 100 µl of substrate solution (TMB, ScyTek Co., Ltd.) was added to each well and the plate was left to stand at room temperature for 10 minutes, and 100 µl of reaction stopping solution (Stopping reagent, ScyTek Co., Ltd.) was added to each well so as to stop the enzyme reaction. The absorbance at 450 nm of each well was measured by use of a microplate reader. The results are shown in Fig. 26. The sandwich ELISA using the rabbit anti-human sOBM polyclonal antibody almost equally detected both of human sOBM (molecular weight: about 32 kDa) and human OBM (molecular weight: about 40 kDa) , and measurement sensitivity was about 12.5×10^{-3} pmol/ml (about 500 pg/ml for human OBM, about 400 pg/ml for human sOBM). It was revealed that measurements of mouse sOBM and mouse OBM by ELISA using the rabbit anti-mouse sOBM polyclonal antibody obtained in Example 28 could be made in the same manner as described above, measurement sensitivity in measuring mouse OBM or mouse sOBM was similar with that in human OBM or human sOBM, and a very small amount of mouse sOBM or mouse OBM could be measured.

As described above, since the present anti-human sOBM polyclonal antibody obtained in Example 28 recognized both of human sOBM and human OBM as antigen equally, it was named an anti-human OBM/sOBM polyclonal antibody. Meanwhile, since the anti-mouse sOBM polyclonal antibody obtained in Example 28 recognized both of mouse sOBM and mouse OBM as antigen equally, it was named an anti-mouse OBM/sOBM polyclonal antibody.

[Example 30]

Preparation of Monoclonal Antibody

The purified human sOBM obtained in Example 28 was used as an immunizing antigen. The purified human sOBM was dissolved in phosphate buffered saline at a concentration of 10 µg/ml. To the prepared human sOBM solution, an equal amount of Freund's complete adjuvant was added so as to emulsify it. Thereafter, 200 µl of the antigen was administered into the abdominal cavity of each Balb/c mouse at

an interval of one week for a total of three times so as to immunize the mice. Then, to a physiological saline solution containing 5 µg/ml of the human sOBM, an equal amount of Freund's incomplete adjuvant was added so as to fully emulsify it, and 200 µl of the emulsion was administered to each of the above Balb/c mice at an interval of one week for a total of four times so as to further immunize the mice. After the passage of one week from the fourth additional immunization, 100 µl of phosphate buffered saline solution containing 10 µg/ml of the human sOBM was parenterally administered to each of the Balb/c mice for booster. On the 3rd day after the final immunization, the spleen was removed, and spleen cells were separated and fused with mouse myeloma cells P3x63-AG8.653 in accordance with a known method (Koehler, G. and Milstein, C., Nature, 256, 495 (1975)). After completion of the fusion, the cell suspension was cultured in a HAT medium containing hypoxanthine, aminopterin and thymidine for 10 days. After the myeloma cells perished and hybridomas appeared, the medium was replaced with an HT medium obtained by removing aminopterin from the HAT medium, and the culture was continued.

[Example 31]

Selection and Cloning of Hybridoma

Since the appearance of the hybridoma was seen on the 10th day from the start of the cell fusion and culturing in Example 30, a high affinity antibody recognizing human sOBM and hybridoma producing the antibody were selected in the means of the following improved solid phase ELISA. Further, to select an anti-OBM monoclonal antibody recognizing both of human sOBM and mouse sOBM, the mouse sOBM obtained in Example 27 as well as human sOBM was used as an antigen in the solid phase ELISA. Human sOBM and mouse sOBM each was dissolved in 0.1 M sodium hydrogen carbonate solution at a concentration of 5 µg/ml, and 50 µl of each antigen solution was added to each well of 96 well immunoplate (Nunc Co., Ltd.) and the plate was left to stand at 4°C overnight so as to solid-phase the antigens. The antigen solution in each well was discarded, and 200 µl of 50% BLOCKACE (Snow Brand Milk Products Co., Ltd.)

was added to each well and the plate was left to stand at room temperature for 1 hour so as to cause blocking. After each well was washed with a phosphate buffered saline containing 0.1% polysorbate 20, 40 µl of bovine serum (HICLONE CO., LTD.)

5 was added to each well. Then, 10 µl of hybridoma conditioned medium was added to each well and the plate was left to stand under a serum concentration of 80% at room temperature for 2 hours so as to cause reaction. An object of the solid phase ELISA in the presence of 80% serum is to select an antibody

10 capable of binding to a small amount of human SOBM or mouse SOBM even in the presence of protein and a serum-derived immune reaction inhibiting substance in high concentration , that is, to select a hybridoma producing an antibody having high affinity for human SOBM or mouse SOBM. After completion

15 of the reaction at room temperature for 2 hours, the plate was washed with PBS-P, and 50 µl of diluent of peroxidase labeled anti-mouse IgG (KPL CO., LTD.) diluted to 5,000 times with a physiological saline solution containing 25% BLOCKACE was added to each well and the plate was left to stand at room

20 temperature for 2 hours so as to cause reaction. After the plate was washed with PBS-P three times, 50 µl of substrate solution (TMB, ScyTek Co., Ltd.) was added to each well and left to stand at room temperature for 5 minutes. Then, 50 µl

25 of a reaction stopping reagent (Stopping Reagent, ScyTek Co., Ltd.) was added so as to terminate the enzyme reaction. The absorbance at 450 nm of each well was measured by use of a microplate reader (IMMUNOREADER NJ2000, NIPPON INTERMED CO., LTD.) so as to select a hybridoma producing an antibody which recognizes human SOBM or mouse SOBM. The hybridomas showing

30 particularly high absorbance (OD_{450nm}) were selected and repeatedly cloned 3 to 5 times by a limiting dilution method so as to established hybridomas producing antibody stably . Out of the obtained hybridomas, hybridomas having higher antibody productivity were selected.

35 [Example 32]

Production and Purification of Monoclonal Antibody

The antibodies obtained in Example 31, that is, the hybridoma producing an antibody which recognizes human SOBM

with high affinity and the hybridoma producing the antibody which has a cross-reactivity with mouse sOBM were cultured, and each hybridoma was implanted in the abdominal cavity of a Balb/c-based mouse which had been given pristane (Aldrich Chemical Co., Ltd.) about a week before, in an amount of 1×10^6 cells/mouse. After about 2 or 3 weeks, accumulated ascites was sampled so as to obtain ascites containing the monoclonal antibody recognized human sOBM or the monoclonal antibody recognizing human sOBM and mouse sOBM. Purified monoclonal antibodies were obtained from the ascites using protein A column (Pharmacia Co., Ltd.) chromatography in accordance with the method for purifying an anti-OBM/sOBM polyclonal antibody described in Example 28.,

[Example 33]

Antigenic Specificity of Monoclonal Antibody

The antigenic specificities of monoclonal antibodies, which specifically recognized human sOBM, and of monoclonal antibodies, having cross-reactivity with human sOBM and mouse sOBM, were examined using human sOBM, intact human OBM having a membrane binding site, mouse sOBM, and ,intact mouse OBM having a membrane binding site, as antigens. Although over 30 types of monoclonal antibodies were obtained, the results of representative monoclonal antibodies are shown in Table 1. As a result, it was revealed that most of anti-human sOBM monoclonal antibodies which specifically recognized human sOBM recognized even intact human OBM having a membrane binding site and did not recognize mouse sOBM and intact mouse OBM having a membrane binding site.

Meanwhile, a few monoclonal antibodies recognizing both of human sOBM and mouse sOBM were also obtained and it was found that these antibodies had cross-reactivity with human OBM and mouse OBM. These results indicate that human OBM and mouse OBM had a common antigen recognition site, i.e., epitope. Since an anti-human sOBM monoclonal antibody prepared by use of human sOBM as an antigen also equally recognized human OBM, which was a membrane binding intact protein, the monoclonal antibody was named an anti-human OBM/sOBM monoclonal antibody.

Table 1

Antibody	Antigen			
	hsOBM	hOBM	msOBM	mOBM
H-OBM 1	+	+	-	-
H-OBM 2	+	+	-	-
H-OBM 3	+	+	-	-
H-OBM 4	+	+	-	-
H-OBM 5	+	+	-	-
H-OBM 6	+	+	-	-
H-OBM 7	+	+	-	-
H-OBM 8	+	+	-	-
H-OBM 9	+	+	+	+
H-OBM 10	+	+	-	-
H-OBM 11	+	+	-	-
H-OBM 12	+	+	-	-
H-OBM 13	+	+	+	+
H-OBM 14	+	+	-	-

(hsOBM: human soluble OBM, hOBM: human membrane binding OBM, msOBM: mouse soluble OBM, mOBM: mouse membrane binding OBM)

[Example 34]

Tests of Class and Subclass of Monoclonal Antibody

5 The class and subclass of the monoclonal antibody of the present invention were determined by use of the Immunoglobulin Class/Subclass Analytical Kit (Amersham Co., Ltd.). The tests were conducted in accordance with a protocol provided in the kit. The results of representative monoclonal antibodies are shown in Table 2. The majority of anti-human OBM/sOBM monoclonal antibodies had IgG₁, and some antibodies having IgG_{2a} or IgG_{2b} were also found. Further, all of the antibodies had κ chain as a light chain.

Table 2

Antibody	IgG ₁	IgG _{2a}	IgG _{2b}	IgG ₃	IgA	κ
H-OBM 8	-	+	-	-	-	+
H-OBM 9	+	-	-	-	-	+
H-OBM 10	+	-	-	-	-	+
H-OBM 11	+	-	-	-	-	+
H-OBM 12	-	-	+	-	-	+
H-OBM 13	+	-	-	-	-	+
H-OBM 14	+	-	-	-	-	+

15 [Example 35]

Measurement of Dissociation Constant (K_d value) of Monoclonal Antibody

20 A dissociation constant of monoclonal antibodies was measured in accordance with a known method (Betrand Friguet et al.: Journal of Immunological Methods, 77, 305 to 319, 1986). That is, the purified antibody obtained in Example 32 was diluted at 5 ng/ml with 0.4 M Tris-HCl containing 40% BLOCKACE and 0.1% polysorbate 20 (pH: 7.4, primary buffer),

and an equal amount of diluent of the purified human soluble OBM (hsOBM) obtained in Example 28, prepared with the primary buffer at stepwise-concentration from 6.25 ng/ml to 10 µg/ml, was added and the solution was left to stand at 4°C for 15 hours so as to bind the monoclonal antibody to hsOBM. After 15 hours, an antibody unbound to hsOBM was measured by solid phase ELISA with solid-phased hsOBM (10 µg/ml, 100 µl/well) so as to calculate the dissociation constant of the monoclonal antibody to hsOBM. Further, the affinity for msOBM of monoclonal antibodies, having cross-reactivity with mouse soluble OBM (msOBM) and hsOBM, was also measured by using msOBM in place of hsOBM at the above-mentioned method. Particularly, The results of particular antibodies, which had high affinity for each of the antigens and were useful in enzymatic immunoassay, binding assay and such, are shown in Table 3.

Table 3

Antibody	Subclass	Antigen	Dissociation Constant	Kd (M)
H-OBM 1	IgG ₁ (κ)	hsOBM	$1 \times 10^{-11} < Kd < 1 \times 10^{-10}$	
H-OBM 4	IgG ₁ (κ)	hsOBM	$1 \times 10^{-11} < Kd < 1 \times 10^{-10}$	
H-OBM 9	IgG ₁ (κ)	hsOBM	$1 \times 10^{-9} < Kd < 1 \times 10^{-8}$	
H-OBM 9	IgG ₁ (κ)	msOBM	$1 \times 10^{-8} < Kd < 1 \times 10^{-7}$	

As a result, it was found that H-OBM 1 and H-OBM 4 which were specific antibodies for human soluble OBM (hsOBM) showed a dissociation constant of 10^{-11} M order, indicating that they had very high affinity for hsOBM. Meanwhile, the Kd value of H-OBM 9 which was an antibody recognizing both hsOBM and mouse soluble OBM (msOBM) was 10^{-8} M order with respect to msOBM and 10^{-9} M order with respect to hsOBM. Further, regarding to H-OBM 13, which was another antibody recognizing both antigens shown in Table 1, the dissociation constants of H-OBM 13 with respect to both antigens were almost identical with those of H-OBM 9, and since both antibodies had the same subclass, a possibility was suggested that they were the same antibody recognizing the same epitope.

[Example 36]

Method for Measuring Human OBM and sOBM by Sandwich ELISA

Using Anti-Human OBM/sOBM Monoclonal Antibody

Sandwich ELISA was constructed by use of the two types of high affinity monoclonal antibodies obtained in Example 35, i.e., H-OBM 1 and H-OBM 4, as a solid phase antibody and an enzyme labeled antibody, respectively. Maleimide Activated Peroxidase Kit (Pierce Co., Ltd.) was used for labeling the antibody. H-OBM 1 antibody was dissolved in 0.1 M sodium hydrogen carbonate solution at a concentration of 10 µg/ml, and 100 µl of the resulting solution was added to each well of 96-well immunoplate (Nunc Co., Ltd.) and the plate was left to stand at 4°C overnight so as to solid-phase the antibody. After the solution in each well was discarded, 300 µl of 50% BLOCKACE was added to each well and the plate was left to stand at room temperature for 2 hours so as to cause blocking. After the blocking, the plate was washed with phosphate buffered saline containing 0.1% polysorbate 20 (PBS-P). Human soluble sOBM and human OBM each were dissolved in 0.4 M Tris-HCl (pH: 7.4) containing 40% BLOCKACE (Snow Brand Milk Products Co., Ltd.) and 0.1% polysorbate 20 (Wako Pure Chemical Industries, Ltd.) (primary reaction buffer) and diluted so as to prepare test samples with various concentrations. 100 µl of each of test samples prepared at various concentrations was added to each well and the plate was left to stand at room temperature for 2 hours so as to cause reaction. Thereafter, the plate was washed with PBS-P, and 100 µl of POD labeled H-OBM 4 antibody diluted with 0.2 M Tris-HC (pH: 7.4) containing 25% BLOCKACE and 0.1% polysorbate 20 (secondary reaction buffer) was added to each well and the plate was left to stand at room temperature for 2 hours so as to cause reaction. After the plate was washed with PBS-P, 100 µl of substrate solution (TMB, ScyTek Co., Ltd.) was added to each well so as to develop color in the wells, and 100 µl of reaction stopping solution (stopping reagent, ScyTek Co., Ltd.) was added to each well so as to stop the enzyme reaction. The absorbance at 450 nm of each well was measured by use of a microplate reader. The results are shown in Fig. 27.

As a result, it was revealed that the sandwich ELISA

constructed by use of the two types of high affinity anti-human OBM/sOBM monoclonal antibodies obtained in Example 35, i.e., H-OBM 1 and H-OBM 4, detected human OBM and human sOBM equally. The measurement sensitivity thereof was about 1.25 to 2.5 X 10⁻³ pmol/ml (about 50 to 100 pg/ml for human OBM having a molecular weight of about 40 kDa, about 40 to 80 pg/ml for human sOBM having a molecular weight of about 32 kDa), and very small amounts of human OBM and human sOBM could be measured by the ELIZA. Hybridomas producing these two types of anti-human OBM/sOBM monoclonal antibodies, H-OBM 1 and H-OBM 4, were named H-OBM1 and H-OBM4, respectively. Further, a hybridoma producing H-OBM 9, the anti-human OBM/sOBM monoclonal antibody which recognized both mouse OBM and mouse sOBM and exhibited osteoclastogenesis inhibitory activity, was named H-OBM9. These hybridomas were deposited with the National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry with deposit numbers FERM BP-6264 (H-OBM1), FERM BP-6265 (H-OBM4) and FERM BP-6266 (H-OBM9) on November 5, 1997.

[Example 37]

Measurements of Mouse OBM and Mouse sOBM Using Anti-Human OBM/sOBM Monoclonal Antibody Recognizing Mouse OBM and Mouse sOBM

Sandwich ELISA using the anti-human OBM/sOBM monoclonal antibody H-OBM9 recognizing mouse OBM and mouse sOBM and obtained in Examples 33 and 35 as a solid-phased antibody, and using the anti-mouse OBM/sOBM polyclonal antibody obtained in Example 28 as an enzyme labeled antibody, was constructed. Mouse OBM and mouse sOBM were diluted stepwise with the primary reaction buffer in the same manner as in Example 35, and the mouse OBM and mouse sOBM were detected in the same manner as in Example 36. The results are shown in Fig. 28. As a result, it was confirmed that the mouse OBM and mouse sOBM could be detected equally by use of the anti-human OBM/sOBM monoclonal antibody H-OBM 9 which recognized the mouse OBM and mouse sOBM. As shown in the results of Example 35, the antibody H-OBM 9 had a high dissociation constant with respect to the mouse sOBM; in other

words, the antibody had relatively low affinity for the mouse sOBM. Thus, the measurement sensitivities of mouse OBM (molecular weight: about 40 kDa) and mouse sOBM (molecular weight: about 32 kDa) by the above ELISA were about 25×10^{-3} pmol/ml (about 1 ng/ml for mouse OBM, about 0.8 ng/ml for mouse sOBM).

[Example 38]

Assay for Osteoclastogenesis Inhibitory Activity of Anti-OBM/sOBM Antibody

It is known that an osteoclast-like cell (OCL) is derived by co-culture of mouse spleen cell and ST2 cell (mouse bone marrow derived interstitial cell) (Endocrinology, 125, 1,805 to 1,813 (1989)). Thus, it was examined whether derivation of OCL was inhibited by addition of an OBM/sOBM antibody to the co-culture. Since mouse OBM was expressed in the co-culture system, antibodies used in this Example were H-OBM 9 and rabbit anti-mouse OBM/sOBM polyclonal antibody recognizing mouse OBM. The OBM antibodies each were diluted stepwise with α MEM containing 10% FCS and added to a 24 well plate (Nunc Co., Ltd.) in an amount of 700 μ l/well, and male mouse spleen cells suspended in the above medium (2×10^6 /ml) were also added in an amount of 350 μ l/well. Then, trypsinized ST2 cells were suspended (8×10^4 cells/ml) in the above medium containing 4×10^{-8} M Vitamin D₃ and 4×10^{-7} M Dexamethasone, and the resulting suspension was added in an amount of 350 μ l/well and the plate was incubated at 37°C for 6 days for culture. After the plate was washed with PBS once, the cells were fixed by mixture of 50% ethanol and 50% acetone at room temperature for a minute. After the plate was air-dried, substrate solution was added in an amount of 500 μ l/well in accordance with a protocol of a LEUKOCYTE ACID PHOSPHATASE kit (sigma Co., Ltd.) and the plate was left to stand at 37°C for 55 minutes so as to cause reaction. By this reaction, cell showing tartaric acid resistant acid phosphatase activity (TRAP activity), which was a specific marker of osteoclast, were stained. After the plate was washed with distilled water once and air-dried, the number of TRAP positive cells was counted. The results are shown in Table 4. As a result, it

was found that both of the rabbit anti-mouse OBM/sOBM polyclonal antibody and H-OBM 9 inhibited derivation of OCL depending on the concentrations of the antibody. It was found that these antibodies had osteoclastogenesis inhibitory activity as in the case of an osteoclastogenesis factor, OCIF/OPG, and was useful as a medicament for treating bone metabolism abnormality.

Table 4

Amount of Antibody Added (ng/ml)	Number of TRAP Positive Multinucleate Cells	
	Rabbit Anti-Mouse OBM/sOBM Polyclonal Antibody	Mouse Anti-Human OBM/sOBM monoclonal antibody (H-OBM 9)
0	1,155 ± 53	1,050 ± 45
10	510 ± 24	650 ± 25
100	10 ± 3	15 ± 4

(average ± standard deviation, n = 3)

[Example 39]

Osteoclastogenesis Inducing Activity of Trx-OBM

Mononuclear cells were prepared from whole blood sampled from a vein of a normal adult human using Histopaque (sigma Co., Ltd.) with density gradient technique in accordance with an attached protocol. The mononuclear cells were suspended at a concentration of 1.3×10^6 cells/ml with α -MEM containing 10^{-7} M dexamethasone, 200 ng/ml of macrophage colony stimulating factor (Midori Juji Co., Ltd.), 10% fetal bovine serum and stepwise concentration (0 to 100 ng/ml) of purified Trx-OBM obtained in Example 15, and the suspension was added to a 48 well plate in an amount of 300 μ l/well and the plate was incubated at 37°C for 3 days for culturing cell. Thereafter, the medium was replaced with new one identical with above and the plate was incubated at 37°C for another 4 days for culturing cell. Cell showing tartaric acid resistant acid phosphatase activity (TRAP activity) were selectively stained by the method described in Example 5, and the number of stained multinuclear cells was counted under the microscope. The results are shown in Fig. 29. As a result, cells showing TRAP activity were hardly detected in the wells containing no Trx-OBM, while TRAP positive multinuclear cells

appeared in a manner depend on concentration of Trx-OBM when Trx-OBM was added. Further, these TRAP positive multinuclear cells showed positive result for vitronectin receptor which was a marker of osteoclast. In addition, when the same

5 culturing was conducted on dentin fragments placed on a 48 well plate, absorption cavities were formed on the surface of dentin fragments only in the presence of Trx-OBM. Thereby, it was revealed that Trx-OBM had activity to induce formation of human osteoclasts.

10 [Example 40]

Bone Resorption Inhibitory Activity of Anti-OBM/sOBM Antibody

To 15-day pregnant ddy mice(Nippon SLC Co., Ltd.), 25 μ Ci of [45 Ca]-CaCl₂ solution (Amersham Co., Ltd.) was injected subcutaneously, and bone of fetus was labeled with 45 Ca. On

15 the following day, the mice were slaughtered, and their abdomens were opened to remove fetuses from the uteruses. A forelimb was removed from the fetus, the skin and muscle were removed to take out a long bone, and a cartilage on the long bone was also removed so as to leave only the diaphysis of the

20 long bone. Each diaphysis was floated in 0.5 ml of culture medium (BGJb medium (GIBCO CO., LTD.) containing 0.2% bovine serum albumin (sigma Co., Ltd.)) and cultured at 37°C in the presence of 5%CO₂ for 24 hours. After completion of the pre-culture, the long bone was transferred to a new culture medium

25 (0.5 ml) containing various bone resorption factors (vitamin D₃, prostaglandin E₂, parathyroid hormone, interleukin 1 α) and, normal rabbit IgG (100 μ g/ml; as a control) or the rabbit anti-OBM/sOBM polyclonal antibody obtained in Example 28, and then cultured for another 72 hours. After completion of the

30 culture, the long bone was put into 0.5 ml of 5% trichloroacetic acid aqueous solution (Wako Pure Chemical Industries, Ltd.) and treated at room temperature for at least 3 hours so as to be decalcified. To the conditioned medium and the trichloroacetic acid extract (0.5 ml each), 5 ml of

35 scintillator (AQUASOL-2, PACKARD CO., LTD.) was added and the radioactivity of 45 Ca was measured, and the proportion of 45 Ca liberated in the culture solution due to bone resorption was calculated. The results are shown in Figs. 30 to 33. As a

result, although the vitamin D₃ (10^{-8} M) caused increase of bone resorption activity, the bone resorption caused by the vitamin D₃ was inhibited by addition of the rabbit anti-OBM/sOBM polyclonal antibody in a concentration-dependent manner, and the bone resorption was completely inhibited by addition of the antibody at a concentration of 100 µg/ml (Fig. 30).

Further, although bone resorption activity was increased in the presence of prostaglandin E₂ (10^{-6} M) or the parathyroid hormone (100 ng/ml), the bone resorption caused by the prostaglandin E₂ or the parathyroid hormone was almost completely inhibited by the addition of the rabbit anti-OBM/sOBM polyclonal antibody (100 µg/ml) (Figs. 31 and 32).

Meanwhile, the normal rabbit IgG (100 µg/ml) used as a positive control had no effects on the bone resorption by the

prostaglandin E₂ and the parathyroid hormone. Further, although bone resorption was induced by the interleukin 1α (10 ng/ml) as well, the bone resorption was inhibited significantly by the rabbit anti-OBM/sOBM polyclonal antibody (100 µg/ml) (Fig. 23). From these results, it was revealed that the antibody of the present invention was excellent as a bone resorption inhibitory substance. As a result of conducting the same experiment on H-OBM 9 which was a mouse anti-human OBM/sOBM antibody, it was confirmed that H-OBM 9 had approximately equal bone resorption inhibitory activity to that of the rabbit anti-OBM/sOBM polyclonal antibody.

Industrial Applicability

The present invention provides a novel protein which binds osteoclastogenesis inhibitory factor (OCIF), a method for production thereof, a method for screening a substance which controls expression of the protein by use of the protein, a method for screening a substance which inhibits or modifies an activity of the protein, a method for screening a receptor which binds the protein and transmits an activity thereof, a pharmaceutical composition comprising a substance obtained by said method for screening, an antibody to the protein, and an agent for treating bone metabolism abnormality which is formulated using the antibody.

Furthermore, the present invention provides a DNA which encodes a novel protein (OCIF binding molecule) which binds osteoclastogenesis inhibitory factor; OCIF, a protein having an amino acid sequence encoded by the DNA and, a method
5 for genetically producing a protein which specifically binds to the OCIF by use of the DNA, and an agent for treating bone metabolism comprising the protein. Moreover, there are provided a method for secreting a substance which controls the expression of the OCIF binding molecule, a method for
10 screening a substance which binds to the OCIF binding molecule and inhibits or modifies an activity thereof, a method for screening a receptor which binds OCIF binding molecule and transmits an activity thereof, and a pharmaceutical composition comprising a substance obtained by said method for
15 screening.

In addition, there are provided a DNA which encodes a novel human protein (human-derived OCIF binding molecule, human OBM) which binds osteoclastogenesis inhibitory factor OCIF, a protein and having an amino acid sequence encoded by
20 the DNA, a method for genetically producing a protein which specifically binds OCIF and has a biological activity to support or promote the differentiation and maturation of osteoclast by use of the DNA, and an agent for treating bone metabolism abnormality comprising the protein.

Furthermore, there are provided a method for screening
25 a substance which controls expression of the OCIF binding molecule, a method for screening a substance which binds the OCIF binding molecule and inhibits or modifies an activity thereof, a method for screening a receptor which binds the
30 OCIF binding molecule and transmits the biological activity thereof, and a pharmaceutical composition comprising a substance obtained by said method for screening, as well as an antibody to the human-derived OCIF binding protein, and an agent for preventing and/or treating bone metabolism
35 abnormality which is formulated using the antibody.

Moreover, the present invention provides an antibody (anti-OBM/sOBM antibody) which recognizes both of the following antigens, i.e., a membrane binding molecule (OCIF binding molecule; OBM) protein which specifically binds to an

OCIF, and, a soluble OBM (sOBM) lacking membrane binding sites
, a method for production of the antibody, a method for
measuring the OBM and sOBM by use of the antibody, and an
agent for preventing and/or treating bone metabolism
5 abnormality which comprising the antibody as an active
ingredient.

The proteins or antibodies presented by the present
invention are useful as medicaments, experimental reagents or
diagnostic reagents.

10 Reference to Deposited Microorganisms

(1) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology
of the Agency of Industrial Science and Technology of the
15 Ministry of International Trade and Industry
1-1-3 Higashi, Tsukuba-shi, Ibaragi-ken, Japan (zip:
305)

Date of Deposit:

May 23, 1997

20 Deposit Number:

FERM BP-5953

(2) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology
of the Agency of Industrial Science and Technology of the
25 Ministry of International Trade and Industry
1-1-3 Higashi, Tsukuba-shi, Ibaragi-ken, Japan (zip:
305)

Date of Deposit:

August 13, 1997

30 Deposit Number:

FERM BP-6058

(3) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology
of the Agency of Industrial Science and Technology of the
35 Ministry of International Trade and Industry
1-1-3 Higashi, Tsukuba-shi, Ibaragi-ken, Japan (zip:
305)

Date of Deposit:

November 5, 1997

Deposit Number:

FERM BP-6264

(4) Name and Address of Depository Institution:

5 National Institute of Bioscience and Human-Technology
of the Agency of Industrial Science and Technology of the
Ministry of International Trade and Industry
1-1-3 Higashi, Tsukuba-shi, Ibaragi-ken, Japan (zip:
305)

Date of Deposit:

10 November 5, 1997

Deposit Number:

FERM BP-6265

(5) Name and Address of Depository Institution:

15 National Institute of Bioscience and Human-Technology
of the Agency of Industrial Science and Technology of the
Ministry of International Trade and Industry
1-1-3 Higashi, Tsukuba-shi, Ibaragi-ken, Japan (zip:
305)

Date of Deposit:

20 November 5, 1997

Deposit Number:

FERM BP-6266